

Earth summit raises expectations

The UN Conference arranged for June is more likely to succeed if over-ambitious planning can be somehow tempered by a sense of what is politically and financially feasible.

THE United Nations, much strengthened in reputation by events of the past two years and, more recently, by the appointment of one good secretary-general to succeed another, should think hard about the conference on Environment and Development, otherwise the 'Earth Summit', to be held in Rio de Janeiro in June. There are two dangers. First, an agenda almost predestined to be over-ambitious is also predestined to disappoint the enthusiasts who will be watching what happens at Rio. Second, to the extent that one objective of the conference is to explore the needs of developing countries for the technical and financial assistance that will enable them to follow commonly determined environmental goals, the conflict (rather than the identity) of interests between developing and developed countries may be rancorously re-advertised. It is in everybody's interest that ambitions for Rio should be curtailed.

The central disagreement about the likely agenda for Rio (being drafted in New York this week and during the next four) is that embodied in the saying that "the best is often the enemy of the good", which means that the pursuit of an ideal may impede, even prevent, the attainment of lesser but worthwhile goals. At one extreme, in the interests of what Adlai Stevenson once called "the care and maintenance of a small planet", there is a plan for asking the governments participating at Rio to sign a declaration tantamount to a recipe for the long-term preservation of the surface of the Earth much as it is now, together with an acknowledgement of the financial obligations to developing countries implied by consequential restraints on development. At the other is the view that Rio should be an occasion for settling a few urgent matters, among which the most urgent is a convention to safeguard against the threat of global climatic change. It will be the better if the second wins support.

The case for a convention on greenhouse gases is urgent because the prospect of global warming caused by the accumulation of carbon dioxide, while not proved to the hilt, is substantial and immediate on the timescale of diplomatic negotiations on such contentious issues. Many other matters being canvassed for Rio as issues in their own right, deforestation for example, are parts of that problem, but should strictly be compared with other contributory causes (the continuing use of chlorofluorocarbons, which are also greenhouse gases) and partial remedies (such as nuclear power). But the

comparison is far from simple. Nobody could now seriously pretend that a greenhouse convention accurately trading off one kind of emission against another could be signed at Rio. The best hope is that participating governments will agree that there is a problem to be solved, and will then establish a mechanism to that end. The worst is that there will be an agreement implying the uneconomic use of resources — and from which many important governments will stand aside.

Sadly, there seems an ambition among the organizers to make Rio an occasion when black sheep will be recognized (and pilloried) as such. The United States, consistently sceptical of global warming, is the most conspicuous. But what purpose would be served if the expected wrangle over a greenhouse convention served further to isolate the government which at present spends most on relevant research, and whose own practices are one of the principal contributors to atmospheric carbon dioxide. (Canada, per capita, is marginally more prolific a source.)

More generally, the preparations for Rio seem to imply that, if only recalcitrant politicians would agree, the world's environmental problems could be settled at Rio once and for all. There may, of course, be value in another comprehensive statement of what these problems are, but who can seriously pretend that enough is yet known of them for remedies to be designed in the next few months? The best course for the time being would be to use this global statement as a means of winning agreement that there needs to be a better mechanism for winning collaboration between governments of problems other than global warming that are certain to arise in the future. Just that, of course, would disappoint the enthusiasts, but if they win more, it will be the worse for the rest of us. □

US biotechnology policy

Products pose no special risks just because of the processes used to make them

THE Administration of US President George Bush has just issued a policy on the regulation of biotechnology that is utterly in keeping with good science. Perhaps it should not be surprising that this is so, but for the past two decades biotechnology has gained such a reputation as a bogeyman of science that it is refreshing to see that clear



thinking prevailed at the White House, where a biotechnology policy has been in the works for more than a year.

The policy, which is meant to inform the way individual regulatory agencies handle biotechnology products, states that "the same physical and biological laws govern the response of organisms modified by modern molecular and cellular methods and those produced by classical methods.... [Therefore] no conceptual distinction exists between genetic modification of plants and microorganisms by classical methods or by molecular techniques that modify DNA and transfer genes."

In short, regulation of biotechnology products, whether in agriculture, medicine, pharmaceuticals or manufacturing, should be based on any inherent risk in the product, not on the process by which it is made.

The US policy also acknowledges one of the important scientific truths about modern biotechnology products. They may be safer than their conventionally derived counterparts, largely because their characteristics—often down to the level of DNA sequences—are so thoroughly known. Concern about the risk of pesticides, herbicides, and other agricultural products made with recombinant DNA technology has overshadowed the fact that conventional agents can also pose unexpected problems. Such is the case now in the state of Florida where thousands of acres of vegetables have rotted because a much trusted fungicide called Benlate, used for two decades by farmers in hot, humid climates, has suddenly stopped working. Nobody knows why.

The White House policy is intended to keep regulatory barriers as low as possible in the US biotechnology industry which, at \$4,000 million today, is predicted to be a \$50,000 million industry by the end of the decade, and thus a significant player in the game of international competitiveness that is one of the President's main concerns. There are already more than 1000 free-standing biotechnology companies employing approximately 70,000 people in the United States, not including the older, established pharmaceutical and chemical companies that are also now in the biotechnology business. The Republicans do not want to see the industry crippled by regulations based on fear rather than a serious assessment of risk.

It is not surprising that biotechnology products, particularly those released into the environment by the agricultural and chemical industries, have elicited strong negative reactions from environmental groups, as well as from ordinary citizens. After all, the very scientists who developed recombinant DNA technology were the ones who alerted the public to its potential hazards, particularly if gene-spliced organisms were to multiply out of control. But 20 years of real-life experimental and commercial science has shown those fears to be largely baseless, while the benefits of the technology (creating herbicide resistant crops, for instance) are easy to identify.

The Administration is in no way suggesting that biotechnology products go unregulated. Rather, it simply says that "products developed through biotechnology processes do not *per se* pose risks to human health and the environment." This approach is entirely sound and long overdue. □

What rate for the job?

The pay of British academics and researchers is scandalously low, but how can it be improved?

If British academics and researchers were realists, they would not let themselves be distracted from their search for jobs elsewhere by the chore of voting in the forthcoming and widely advertised general election. Whichever party forms the next government, the lot of the British research profession will not be substantially improved. True, both the government party and the Labour opposition promise to improve access to an education in science for young people, but neither party is promising to put right the abiding scandal in British universities and research laboratories — the general impoverishment that stems from low salaries.

For the past decade, the growth of academic and research salaries has been linked with inflation, not general prosperity (see *Nature* 353, 105–112). A letter on page 10 cogently argues that the consequences of this state of affairs damage British science as a whole. Who can expect the research profession to compete successfully for new recruits in a dwindling age group when the prospects are known to be so poor? Professor Howard Morris rightly complains that the leaders of the profession have paid too little attention to the issue. His remedy is that the Committee of Vice-Chancellors and Principals (loosely representative of academic institutions) should wash its hands of pay negotiations and demand an independent review instead.

That would be a good start; other British public servants (judges, teachers, nurses and policemen) have recently done rather well from pay boards. But proper rates of pay for academics and academic researchers raise more complicated problems. Not the least of these is the British convention that rates of pay for people of specified age and seniority should be determined by national negotiations. For the past decade, the government has been trying to break this convention, most conspicuously by its ham-fisted threat that universities failing to pay some professors more than others would be denied an increase of recurrent funds. But now that the British university system is being impelled in a direction in which there will be a distinction between research and teaching institutions, is it not in the interests of universities themselves that salaries should be determined institutionally, not nationally?

Academics do not fully appreciate the importance of such derogation as an ingredient of cherished academic freedom. The most obvious benefit, that successful institutions may be able to recruit able people by offering them internationally competitive salaries, is not the most important. What of an unsuccessful institution, believing that its virtues will eventually shine through, that may decide that its best chance of survival is that its members should pay themselves less? That is the direction in which British universities and research laboratories should now be pushing. Of course, nothing can be done until after the general election. Will the climate be any better afterwards? □

Researchers criticize NIH response to PETA claims

- Agency closed laboratory within a day
- Investigation finds only "minor problems"

Washington

RESEARCHERS at the US National Institutes of Health (NIH) are up in arms about an incident last fall in which the agency suspended the experimental protocols of a laboratory less than 24 hours after receiving what later turned out to be a largely unsubstantiated complaint by animal activists.

Last week, a subcommittee of the NIH Animal Care and Use Committee finished a four-month investigation of the case. In the subcommittee's report, which has not yet been released, the investigators are said to have found some violations of record keeping and procedural rules within the laboratory. But the report finds no reason to suspend the experimental protocols. The protocols were reinstated in late December.

NIH officials are now reviewing their handling of the case. And the agency has set up a special committee to help it make better decisions the next time an allegation is raised.

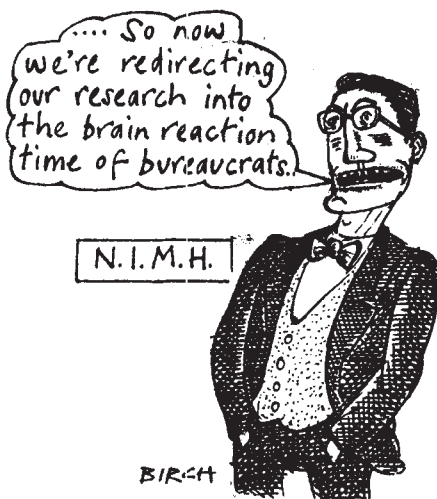
The laboratory in question is run by Josef Rauschecker, a German brain researcher now with the National Institute of Mental Health (NIMH). In December 1989, he and three graduate students from the Max Planck Institute for Biological Cybernetics in Tübingen came to NIH's Poolesville, Maryland, facility with 48 cats, many of which had been surgically blinded and implanted with electrodes. Rauschecker is investigating neural plasticity, the ability of the brain to reorganize itself after an injury.

The conditions of the animals caused "some consternation" when they arrived, says John Miller, director of the animal welfare division in the NIH Office of Protection from Research Risks (OPRR), because of their previous surgery and a respiratory infection that many had developed during travel. The cats were inadvertently allowed to breed during quarantine, and most of the kittens died. Many of the cats also tested positive for antibodies to feline infectious peritonitis (FIP), a virus that is usually fatal. Mortality rates were relatively high, Rauschecker says, averaging about 30 per cent for kittens and 10 per cent for adults, and peaking during a one-month period in August 1991 when there was no veterinarian at Poolesville.

Staff at the facility are said to have been concerned about an unusually high mortality rate during certain experiments and the fact that Rauschecker had not obtained

a breeding permit. Eventually one of them called People for the Ethical Treatment of Animals (PETA).

On 14 November, PETA faxed a complaint to Bernadine Healy, the NIH director, recounting the allegations of abuses in Rauschecker's laboratory. Mary Beth Sweetland, a PETA investigator, wrote that the employee had said that all the cats were infected with FIP on arrival, and had been allowed to live only so that Rauschecker could continue his experiments. (According to the NIH investiga-



tors, most of the cats do not appear to have actually been carrying FIP, although they may have been exposed to the disease at some point.) PETA expressed its hope that Healy would give the matter her "immediate attention".

To the surprise of nearly everyone, that is exactly what happened. Within hours of receiving the fax, Healy had directed Steven Paul, director of the NIMH intramural programme, and Robert Whitney, director of the animal care committee, to investigate the matter. By the next day, she had called in the OPRR as well, "to accelerate the review", according to a response to PETA on 15 November.

Even PETA was flabbergasted by the response. "It almost made me suspicious that someone had known beforehand" about problems with Rauschecker's laboratory, says Sweetland. In fact, there had been a complaint from an employee about animal welfare in the laboratory, says Ronald Schoenfeld, deputy director of intramural research at NIMH. But it had concerned a veterinarian's treatment of a primate, not cats, and had been handled appropriately, he says. At NIH headquar-

ters, PETA's complaints were new.

As NIH officials rushed to respond to the allegations, almost the only person they did not question was Rauschecker himself. He and several members of his laboratory were at the annual meeting of the Society for Neuroscience in New Orleans. When Rauschecker returned to his laboratory on 17 November, he learned that NIH had suspended his protocols.

But within a week, OPRR had bowed out of the case. Miller says that he found no reason to depart from the usual procedures, which involve an initial investigation by the animal care committee followed by a review by the intramural research director and only then by OPRR. A week later, NIMH officials recommended that the protocols be reinstated. But because the request had to be approved by the NIH animal care committee as well, the actual lifting of the suspensions took another three weeks. During the suspension period, laboratory researchers were forced to halt several experiments because they were not allowed to take regular readings from instruments to which the cats were connected.

NIH officials defend their initial measures as a prudent response to a potentially serious complaint. They say they could not at first decide whether the PETA complaint had merit because Rauschecker was not available and Poolesville is several miles from the main NIH campus. "If it had been someone in a facility next door who we could walk down to and check on," says NIH's Schoenfeld, "the response might have been different".

But some NIH researchers see it differently. They are concerned that NIH overreacted to the complaint to avoid a potential public relations disaster. "I was very disturbed when I heard that allegations from a animal rights organization had resulted in a protocol being suspended," says Frederick Miles, an animal care committee member. "PETA's in the business of shutting down research. If we're not careful, the [interests of the] scientist will be left out of this entirely."

PETA's letter to Healy appears to be the first complaint about intramural research to go straight to a NIH director. "It caught everyone by surprise," Miles says. "We were very unprepared." But its success makes it likely that animal rights activists will use the tactic again.

With that in mind, the NIH animal care committee has set up a subcommittee, chaired by Miles, to provide a mechanism to avoid confusion in the future. One potential solution, Miles says, is to appoint an NIH 'ombudsman' who could order a fact-finding investigation within hours of receiving an allegation. With veterinarians and other experts on call, a 'quick response team' would visit the implicated laboratory and decide within 24 hours what to do next.

Christopher Anderson

Pentagon keeps its fruits to itself

Washington

THE US Defense Department is ignoring the intent of a government programme created nine years ago to turn laboratory discoveries into commercial products by insisting that most of its awards be used to make things that the military can use.

The General Accounting Office (GAO) reported last week that a majority of the nearly \$200 million in sales by companies funded by the Defense Department under the Small Business Innovative Research (SBIR) programme have been made to the government, not to the private sector. By contrast, companies funded by the SBIR programme within the National Institutes of Health generate 90 per cent of their revenues from commercial sales. The GAO report questions the Pentagon's "commitment to the goal of increasing private-sector commercialization".

Each federal agency that spends more than \$100 million on research must devote 1.25 per cent of its research budget to the SBIR programme. Some five agencies — defense, energy, health and human services, the National Aeronautics and Space Administration (NASA) and the National Science Foundation (NSF) — provide more than 90 per cent of all SBIR funds. The \$250 million a year spent by the Defense Department represents slightly more than

half of the total.

James Browning, the president of Browning Thermal Systems, a small New Hampshire company that makes high-powered thermal spray paint guns, offered a graphic example of the military's

his entrepreneurial instincts in pursuing his ideas. Those instincts, he says, have generated millions of dollars from patent licences as part of a \$160-million industry. By contrast, he says, an SBIR grant his company received from the Defense

Advanced Research Projects Agency (DARPA), for drilling into crystalline rocks to great depths, was terminated after its initial phase because DARPA had learned what it needed to know.

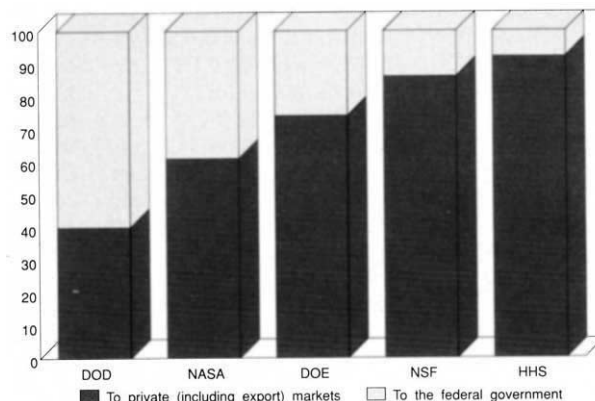
A Pentagon official defended the military's emphasis on 'mission-oriented' research. Ray Wrenn, who runs the SBIR programme for the Defense Department, said that comparisons between his agency and basic research agencies such as NSF and NIH are "inappropriate and misleading". Wrenn also said that many of the Pentagon's SBIR grants go to "leading-edge technology where the commercial market has yet to be established".

Keith Fultz, who directed the GAO study, told the congressional panel that one way to promote commercial sales is to encourage the Pentagon to support technologies that can be used by both the military and the civilian sector. He pointed to several examples where SBIR projects funded by the Pentagon generated sales to both sectors. But he appeared sceptical that the department would loosen its strict rules about the applicability of projects to military needs.

Jeffrey Mervis

Sales of SBIR-funded companies

percent of sales



Source: General Accounting Office

approach to SBIR during testimony last week to the technology subcommittee of the Science, Space and Technology Committee of the US House of Representatives, which requested the GAO report. The subcommittee is considering whether to renew the programme, which expires in 1993.

Browning says that his company was founded on SBIR grants from NSF, which gave him almost total freedom to follow

JAPANESE COLLABORATION

The rising price of helping Bulgarian science

Tokyo

A JAPANESE researcher, with the help of Japan's usually stodgy bureaucracy, has kept alive for a year a whole laboratory of Bulgarian scientists and technicians. But his creative use of a mere US\$50,000 from his large government grant may be foiled by the rising price of freedom in Eastern Europe.

Kuniaki Nagayama, a researcher at JEOL Ltd., a manufacturer of advanced microscopes, received an 'SOS' in December 1990 from Ivan Ivanov, head of the Laboratory of Thermodynamics and Physico-chemical Hydrodynamics at the University of Sophia in Bulgaria. Ivanov wrote that his team of researchers was on the verge of breaking up because of a lack of funds and a brain drain following the political upheavals in Eastern Europe.

Nagayama decided that Ivanov's team could make a significant contribution to his 'Protein Array' project funded by the Exploratory Research for Advanced Tech-

nology (ERATO) programme of the Research and Development Corporation of Japan (JRDC).

With the help of JRDC officials, Nagayama bent the rules governing ERATO projects to provide Ivanov's team with a small but significant sum of money. The \$50,000 was channelled to Ivanov's laboratory under a system normally used for contracting out data analysis to private companies or other research organizations. It has been enough to hold together Ivanov's team of 18 scientists and technicians for the past year. In addition, two researchers from Ivanov's team have spent a year in Japan under the ERATO grant.

The system of support set up by Nagayama has turned out to be a cost-effective way of carrying out research that, at the same time, supports science in Eastern Europe. Genya Chiba, vice president of JRDC, says that it costs almost as much to bring one Bulgarian scientist to

Japan for a year as to support the whole of Ivanov's laboratory in Sophia University for the past year. But it is becoming more expensive by the day.

Bulgaria suffered a sharp rise in prices last year following moves towards a more free-market economy. Ivanov wants almost twice as much money next year so that he can pay higher salaries to his team. Although plenty of money exists within Nagayama's ERATO grant to cover the request, it would be an unprecedented amount of money for outside, contracted research. A decision is expected soon.

In the long run, Nagayama hopes that the laws governing the ERATO programme will be changed to make it easier to provide ERATO funds to research teams overseas. And he hopes his small initiative will serve as a model for Western nations and Japan to help maintain the research infrastructure in Eastern European countries and the former Soviet Union.

David Swinbanks

Psychologists rethink Burt

London

THE British Psychological Society, which 12 years ago decided to support allegations of scientific fraud against the late Sir Cyril Burt, the author of controversial publications on the inheritance of intelligence, last week announced that it holds no official opinion on the matter. Its decision baffles supporters of Burt, who have been pushing to exonerate him, and seems certain to open a new chapter in the controversy surrounding one of the few documented cases of alleged scientific misconduct in Britain. However, it seems unlikely to shed new light on the debate over the roots of intelligence.

The allegations of fraud were first published by the *Sunday Times* in 1976, five years after Burt's death. The article alleged that Burt had fabricated data in studies said to demonstrate high correlations between the IQ scores of monozygotic twins, raised apart. Burt was also accused of listing research assistants who did not exist.

Ten fellows of the British Psychological Society, led by the retired child psychologist Bill Wall, have been pressing for a full inquiry into the matter. Late last year, Wall and his colleagues appeared to be making progress when the society announced that it intended to reconsider its position on Burt (see *Nature* 354, 97; 1991).

But last week, Wall described the society's new statement as "remarkable for its illogicalities and evasiveness". The confusion, he speculates, comes from an attempt to paper over disagreement within the governing council of the society by crafting a statement that all its members could support. Wall expects the ten dissident fellows to meet shortly to consider their next move.

The group is unlikely to let the matter lie. Robert Joynton, the author of a book that sought to exonerate Burt (see *Nature* 340, 439; 1989), says he will not be satisfied until the society announces that it has changed its mind about Burt's guilt.

Joynton wants the society to reexamine the evidence — something it did not do in producing last week's statement. But as the society is unwilling to do so, Joynton says he would be "very happy" to see another organization launch a separate inquiry. He suggests the British Academy, of which Burt was a member.

Fraser Watts from the Medical Research Council's Applied Psychology Unit in Cambridge, who is president of the British Psychological Society, believes a new inquiry would be inappropriate. "The society never actually held an inquiry of the type that the defenders of Burt are calling for now," he says. (The society assumed that Burt was guilty on the basis

of the conclusions of Burt's biographer, Leslie Henshaw.) Watts is also bemused by the assumption among Burt's supporters that a new inquiry would exonerate him.

"Some of the case against Burt has been punctured," he concedes, but "I'd be very surprised if [an inquiry] would be conclusive". Although evidence has now emerged to suggest that the 'missing' research assistants actually existed, many psychologists are still sceptical of some of Burt's data.

Peter Morris, vice-president of the society from the University of Lancaster, rejects the accusation that the society is evading the issue. The society's council genuinely believes that it should not take a corporate view on the conduct of deceased members, he says, so it would be wrong to compound the error of judging Burt in the past by repeating the process now.

"I guess my view is that two wrongs don't make a right," says Morris. The society intends in December to hold a symposium on the Burt affair at its annual conference in London, but Watts says that no official conclusions will be drawn. The society does not believe it should pass judgement on alleged cases of scientific fraud involving deceased members, says Morris, unless the data in question are used in a way that threatens public health or safety.

Leon Kamin, whose examination of Burt's findings stimulated the original *Sunday Times* article, believes the question of Burt's guilt is of secondary importance to the issue of whether his findings can still be considered a serious contribution to the scientific literature. Kamin, a psychology professor at Northeastern University in Boston, Massachusetts, maintains that there are "too many internal inconsistencies, impossible consistencies, and too much lack of documentation" for Burt's publications to carry any real weight.

Other studies (notably the 'Minnesota Twin Study', published in *Science* 250, 233; 1990), have also found that a high proportion of the variance in IQ is associated with genetic variation. But Kamin says that Burt's data were unique in their scope. If Burt's findings could be taken at face value, he says, "the argument was over".

Sandra Scarr, a psychologist at the University of Virginia, disagrees. "If you eliminate Burt's data, the story and the picture do not change", she says.

Researchers may argue about whether Burt's findings will endure. But one thing is certain: the controversy surrounding them refuses to go away.

Peter Aldhous

Science city launched

Sydney

THE Australian government announced last week that it will spend \$52 million on two technology projects to help its industry compete in the world economy. One will create a 'science city' along the lines of those in Japan, and the other will support a company to commercialize discoveries made in Australian laboratories.

As a trade-off for these two ventures, the government said that it would reduce tax concessions to high-technology companies. Instead of being permitted to write off 150 per cent of their research and development costs from taxable income, companies will be allowed only 125 per cent, starting in the next fiscal year.

Reaction to the government's actions has been mixed. While the new city, called a multi-function polis, is generally considered to be a good idea, Australian university officials have said that they do not need any help in bringing new technology to the market. A committee of them have already complained to the government about its decision to invest \$20 million in the idea, called the Australian Technology Group. And industry is naturally unhappy with having to pay more taxes.

The new city, to be built on the fringes of the South Australian state capital of Adelaide, will be designed for both the people and the research organisations that will be located there. Its planners have said that they want to concentrate on environmental research, information technology and education reform.

The federal government, which has already spent \$9 million on the city, will contribute another \$41 million over the next several years. The federal money will be used to build access and internal roads, as well as a network of waterways to overcome the site's swampy conditions. The state government is expected to chip in another \$75 million over the next ten years.

A federal environmental research group has already been told that it is being moved to the new city. Other federal and state agencies are likely to follow, and Australia's largest company, BHP, is expected to move at least some of its operations there.

Despite the enthusiasm of the government for the project, private investors have been reluctant to commit any of their money or resources. A Japanese business delegation that visited the site recently said, in effect, that there would be no Japanese investment in the project. Japan has created more than a dozen such cities in the past decade, but even the most successful took several years to blossom.

Initial work on the site, projected to become eventually a city of 40,000, is expected to begin before the end of the year. The first stage includes construction of three villages, with housing for 8,000 peoples.

Mark Lawson

NIH aims at 'glass ceiling'

Washington

CONCEDING for the first time that women's health cannot be improved without also improving conditions for women scientists, the US National Institutes of Health (NIH) is expanding its new women's health initiative to try to topple some of the barriers that women face in research.

This week NIH held a two-day hearing on the problem of the 'glass ceiling' — the invisible wall of discrimination that many women scientists run into in their researcher careers. Earlier in the month, NIH released a study on women researchers* showing that the percentage of women applying for biomedical grants is growing

NIH project grants, 1990		
	Men	Women
Applications	13,420	3,135
Success rate	23.6%	23.2%
Average grant size	\$206,000	\$177,000
Priority score (lower scores better)	237	241

Source: NIH Division of Research Grants

although men, on average, still receive larger grants. And in June, the NIH Office of Research on Women's Health will hold a workshop to propose new programmes to help women scientists, a move that marks a new direction for an office that only a year ago was refusing even to consider the issue.

Credit for the shift goes to a 1990 study by the congressional General Accounting Office (GAO), which found women badly underrepresented in NIH-sponsored clinical trials. That, along with a string of women's health scandals such as the Dalkon Shield and breast implants, prompted legislators to give the agency more than \$30 million last year for new research on women's health.

Although NIH had hoped to keep professional issues out of the new programme, the first public meeting of the Office of Research on Women's Health, held last September, demonstrated that the matter could not be ignored. Despite warnings that the meeting should be concerned solely with health research, virtually all the questions and comments were about the problems faced by women researchers, says Ruth Kirschstein, director of the NIH Institute of General Medical Science and the former acting director of the women's health office.

"They were quite clear," Kirschstein says of those who attended that meeting. "As long as there are not enough women doing biomedical research, there will not be enough emphasis on women's health — they go hand in glove."

Vivian Pinn, who last fall became director of the NIH women's health office, says that improving the lot of women researchers has become an "implied mandate" for the office. "If we want women's health research to become institutionalized, we need to move more women researchers into the position of saying what's done."

Women still have a way to go, she points out. Although they make up 39 per cent of all medical school students, women account for only 21.5 per cent of the faculty of those schools and just four per cent of medical school department chairs.

Pinn hopes that this week's hearings and the workshop in June will produce several concrete suggestions on how to increase those numbers. One idea already under consideration is a new tenure system that takes child-bearing and family care into account. Her office is also thinking about creating a national database of available faculty positions and research opportunities to give women researchers a fairer shot at jobs and grants.

Although there is now plenty of money for research on women, no NIH grants are targeted for women researchers themselves. Even a \$500 million, ten-year study on women's health is controlled mostly by the individual NIH institutes, rather than the women's health office. Nevertheless, Pinn says, direct funding for women researchers is "something we can argue for."

Another issue facing the NIH is their own treatment of women. Last year, 194 complaints of sexual discrimination and other gender-related mistreatment were filed against the institutes, according to Self Help for Equal Rights (SHER), an independent organization campaigning for the rights of women and other employee of NIH. At least four lawsuits charging sex discrimination or harassment at NIH are pending.

Pinn acknowledges the problem, but says it is primarily an issue for the agency's equal employment officials. "We have to discuss issues of sexual harassment and gender discrimination [at NIH], but that is not our main focus," Pinn says.

Those issues nevertheless may surface within the women's health programme. Although SHER president Billie Mackay applauds Bernadine Healy, the NIH director, for expanding the scope of the programme, she promises more public battles with the agency as long as its conduct generates lawsuits. Despite the NIH's newfound sensitivity, SHER claims, the agency still has "harassers and discriminators" in virtually every branch of its management tree.

Christopher Anderson

* Women in NIH Extramural Grant Programs: 1981-1990; NIH Division of Research Grants, 1992.

Goodwin stumbles

Washington

A FEDERAL health administrator has been demoted after members of Congress condemned his public comments comparing the conduct of young, inner-city minority males to the behaviour of rhesus monkeys in the wild. But the drama may not be over: questions remain about the wisdom of his running the agency that funds much of that research, and critics want him removed entirely from public office.

Frederick Goodwin, for the past three years the administrator of the Alcohol, Drug Abuse and Mental Health Administration (ADAMHA), resigned last week after apologizing for drawing parallels between hyperaggressive and hypersexual behaviour among male primates and the rising violence among inner-city youths as a result of what he called "a loss of civilizing factors".

Goodwin was immediately named director of the National Institute of Mental Health (NIMH), one of the three institutes that comprise ADAMHA. Goodwin said in his letter of resignation to President Bush that he was stepping down because of his wish to "defuse" an issue that "is being exploited politically". Goodwin's boss, Louis Sullivan, secretary of the US Department of Health and Human Services, said last week that he was speeding up a transition planned for later in the year.

The change is a bit more hasty than Goodwin and Sullivan suggest. Goodwin was to take over NIMH as part of a reorganization of federal health agencies, with the research components of NIMH and its sister institutes of drug abuse and alcohol abuse being subsumed by the US National Institutes of Health. But such a reorganization now appears unlikely. It is opposed by the chairmen of the two committees in Congress that oversee these agencies — both of whom have questioned Goodwin's fitness for continued government service.

Scientists who rely on NIMH for funding are concerned about whether congressional unhappiness with Goodwin will cut into support for the agency's scientific agenda. "How can he be an advocate for his agency's budget if he couldn't be effective one step higher up?" wonders Alan Kraut, executive director of the American Psychological Society.

Researchers are also troubled by the way Goodwin depicted the underlying research. Felton (Tony) Earls, a Harvard psychiatrist who is a member of the NIMH advisory board at which Goodwin made his remarks last month, says he was "shocked" by Goodwin's description of such aggressive behaviour by primates as normal. Another board member, Dominick Purpura of the Albert Einstein College of Medicine, says that Goodwin "knows better than to forget about five million years of evolution."

Jeffrey Mervis

Can China keep its end up?

Hong Kong

WESTERN companies are concerned about China's ability to enforce a new agreement with the United States on intellectual property rights that takes effect this month.

The agreement, part of a memorandum of understanding (MOU) signed by the two countries on 17 January, strengthens Chinese laws in exchange for ending a special investigation by US officials into its trade practices. Among other things, China has agreed to join the Berne Copyright Convention and the Geneva Phonograms Convention within 18 months, as well as to move quickly to pass laws that would expand protection of copyrights, patents and trade secrets. The laws would also apply to such intellectual property owned by foreigners.

But Western lawyers are worried about the vague language in the MOU. The English version of the document is filled with such phrases as "China will use its best efforts" to protect foreign works and will act "within a reasonable period of time". Jeffrey Siebach, regional representative for the Business Software Alliance based in Washington, DC, says that "the variables really can be quite daunting. It's economically unacceptable uncertainty".

Observers also wonder if Chinese officials, both in the capital and throughout the provinces, are prepared to enforce the

new rules. One major question is which ministry will be responsible. Most multinational companies want it to be the National Copyright Administration, which is thought to be impartial. They fear that if the job goes to the Ministry of Machine Building and Electronics Industry, which in the past has taken a protectionist attitude toward the issue, it could sabotage any chance of legislation.

At the same time, the industry ministry has more technical expertise to administer the new rules and more power over Chinese software companies. That knowledge could be very helpful, says Joseph Simone, a Hong Kong lawyer and specialist in Chinese investment. Simone and other lawyers representing Western software companies discussed the matter last week with ministry officials in Beijing.

Another problem is that infringements of patents and copyrights are covered by China's civil code, and not its criminal code. That situation forces aggrieved companies to take their complaints to local authorities. Although some US software companies have said they want to act quickly to protect their patents, they must first find out where to bring their case. In addition, the costs of litigation could well exceed the losses from piracy. Simone, for one, suggests that Western companies would do better to ask local authorities to

negotiate settlements than to take their complaints to the courts.

Chinese and Western sources disagree about the amount of money at stake. US industry claims that it loses more than \$300 million a year as a result of Chinese theft of copyrights. Software companies alone estimate their annual losses at \$160 million, on the basis of the difference between actual and expected sales to a country with half million personal computers.

Zheng Songyu, general manager of the China Patent Agency, a joint venture between Hong Kong and China, thinks that those figures are greatly inflated. He concedes that Chinese companies have copied Western software, but he argues that they have done so mostly for their own use, rather than for commercial gain. He estimates that \$30 million would be a more accurate figure on annual losses to Western companies.

In a speech last December, Zheng predicted that it would take China up to five years to achieve full protection for intellectual property rights. Thanks to the MOU, he says, that process should move ahead much more quickly. **Peter Gwynne**

INTELLECTUAL PROPERTY

US and India in row

Washington

A NINE-MONTH investigation by the United States into Indian patent and trademark policies has found gaping holes in its laws and policies and invoked threats of possible trade sanctions. US officials found that India provides no patent protection for pharmaceutical products and only a very short protection period for other patents, and that foreign patent holders are required to license their products to Indian companies.

US pharmaceutical manufacturers claim that the Indian rules and "rampant piracy" cost them \$200 million in lost sales in India. Indian companies also sell another \$200 million of pirated drugs to other developing countries, according to the Pharmaceutical Manufacturing Association.

Prompted by the US investigation and its own internal inquiry, the Indian parliament is considering a measure to improve enforcement of copyrights and has started compiling data on copyright abuse. The government has begun to train policy officers and prosecutors in copyright law. India also has promised that foreign holders of trademarks will be given the same status as Indian nationals.

US officials are resisting pressure from US pharmaceutical makers to seek "retaliation" for India's current policies. US trade representative Carla Hills, who is negotiating with the Indian government, said she was "leaving the door open" in the hope that India will make positive changes.

Christopher Anderson

CONSERVATION

Red tape delays funds for Amazonia

São Paulo

THE presidents of six Amazonia countries have once again complained that the industrialized nations must do more to stop emissions of greenhouse gases and must spend more on efforts to achieve sustained development of the region. But what the leaders did not talk about at their meeting last month in Manaus was how a considerable amount of money for research and environmental protection already committed by the industrial world is being held up by their own bureaucratic inefficiencies.

"We want access to clean technologies and other means of protecting the environment," said Brazil's president, Fernando Collor de Mello. He failed to mention, however, that his country paid more than \$1 million in fines last year because it failed to use \$150 million lent to it in 1989 by the World Bank to study and protect the Atlantic forest, the Pantanal wetlands and the Amazon rainforest. The government was penalized for failing to pay its share of the multi-million dollar programme. He also kept silent about a German-funded project to protect the Atlantic forest that is stalled in his country's Congress.

But the environment isn't the only loser. Two research institutes, the National Institute for Amazonian Research (INPA) in Manaus and the Belén-based Emilio Goeldi Museum, have waited several months for delivery of some \$80 million promised by the G-7 group of industrial countries that require some matching funds from the Brazilian government. But those funds cannot flow until politicians resolve the country's annual budget battle.

In the meantime, research staffs are being starved for funds. "We do not even have Xerox machines, complains Philip Fearnside, an INPA researcher, "because the institute cannot pay the rent."

INPA's director, Eneas Salati, agrees that the institute may not have money to pay its telephone bills and that office machines must be turned off periodically to conserve a dwindling budget. "It's the beginning of the year, and the budget has not yet been allocated," he says. The interest on the \$50 million over five years that the G-7 nations have pledged would be a significant boost to his institute's current annual budget of about \$10 million. But Salati has no idea when the money is likely to turn up. **Ricardo Bonalume**

Ways to make the West pay

Nairobi, Kenya

A CAMPAIGN to save the dwindling herds of African elephants may do more than protect an endangered species. For more than 100 scientists who met in Nairobi at the end of January, their wildlife conservation efforts are also teaching them how to play the funding game, Western-style.

The scientists arrived at a meeting sponsored by the United Nations Environmental Programme (UNEP) with hopes of tapping into some \$300 million that Western nations have made available for el-



The Mpala Ranch in central Kenya is the latest example of international cooperation to preserve wildlife.

ephant conservation projects. Many expected to leave with money in hand. Instead, except for an announcement by the French government that it would immediately allocate \$10 million for regional conservation, what the scientists received was a better understanding of how to tailor their requests to satisfy their benefactors.

They learned, for example, that they must first do environmental impact studies of how their conservation efforts will affect the surrounding territory. They must show how their survival programmes will work in harmony with various rural development projects already under way. Finally, they must gain the support of their own governments for their plans.

"They must sort out how to present their proposals," says Mona Bjorkland, a senior programme officer for UNEP. "They have to formulate long-term and short-term objectives and justifications, and indicate clearly what they want to get out of the project. They also need a timetable, work plan and cost estimates."

Richard Leakey, director of the Kenya Wildlife Service, believes that success will come from cooperative ventures between African and Western nations. Although environmental officials acknowledge that the West must foot most of the bill, Leakey says that the two sides must be seen as equals if the ventures are to work smoothly.

"As an African country, you come from a position of weakness," he says. "Until African nations make science a higher priority, it will be difficult [for them] to gain an equal footing" in such cooperation ventures.

Leakey says that a proposal to provide money for environmental impact studies as part of other grants made to the developing world will be presented at the United Nations' Earth Summit in Rio de Janeiro this summer. African scientists will find it easier to compete for international funding if these statements are part of their proposals, he says.

Their requests must also be submitted in language that donors are accustomed to reading, says Bjorklund, and channelled through the appropriate embassies or through such multinational organizations as the World Bank, UNESCO or the Food and Agriculture Organization.

They must also persuade their own governments to support them. Wildlife preservation has an understandably low priority in countries faced with such staggering problems as widespread disease, high illiteracy rates, inequitable distribution of land and a lack of food and water. One strategy that might appeal to financially strapped governments is to look at wildlife as a legitimate natural resource that can attract outside investors, says Mark Stanley Price, director of African operations for the African Wildlife Foundation in Nairobi. If properly cared for, he says, wildlife also has the advantage of being a renewable resource.

Perhaps the most unpleasant lesson that the African scientists learned at the meeting was that the funding process can take months or even years. It is unlikely that they will see any of the \$305 million that they have requested, for some 325 projects, before the end of the year. "You can't expect a different set of rules just because it involves elephants," says Price.

A case in point was Leakey's plea at the meeting for surplus material from the United States as it dismantles its military forces in Europe and elsewhere around the world. A representative from the US Fish and Wildlife Service, Ken Stansell, says that his government is more than willing to provide such material as it becomes available, but that there are many requests for it from humanitarian groups as well as from conservationists. In 1990 wildlife officials even convinced Congress to add environmental purposes to the list of acceptable uses of such excess military equipment, he says, and last spring 41 pickup trucks, ten years old but still in working order, were sent by barge to eight African countries after US forces in Germany no longer needed them.

"We have requests in the millions of dollars for this material", says Stansell. "And even though the articles themselves are free, somebody has to pay for whatever repairs, maintenance and shipping costs are incurred."

Jane Stevens

Wellcome diversifies

London

BRITAIN'S largest charitable research foundation, the Wellcome Trust, has decided to sell a large proportion of its 73.6 per cent holding in the drugs company Wellcome plc.

The Trust's move to diversify its assets should provide a short-term boost to its spending on medical research. Even more significantly, the diversification will protect the trust's income from harm if the stock price of the pharmaceutical company drops suddenly. The Trust, which will spend some £80 million on medical research in 1991-92, is the second largest source of funds for British academic biomedical researchers, lagging only behind the government funded Medical Research Council (MRC).

Ninety six per cent of the Wellcome Trust's income comes from its holding in Wellcome plc. "We've got all our eggs in one basket," says Bridget Ogilvie, director of the Trust. "No matter how good that basket is" at present, she says, diversification is essential to guarantee the Trust's future income.

Although Wellcome plc is currently performing well, Ogilvie is well aware of the potential hazards if a charitable foundation fails to spread its assets over a range of quoted companies. "Everyone in Britain knows what happened to Nuffield," she says. Her reference is to the Nuffield Foundation, which saw its income reduced by almost 90 per cent in the early 1970s as its shares in the British Leyland motor company plummeted in value.

If the Wellcome Trust suffered a similar decline in income to that experienced by Nuffield, the consequences for British medical research would be disastrous. Unlike the other UK charitable foundations that live off an invested endowment, the Wellcome Trust's spending is almost entirely concentrated on medical research.

Wellcome's trustees want to reduce the Trust's holding in Wellcome plc to between 25 and 50 per cent of the company's shares. This will need court permission, as the will of Sir Henry Wellcome and rules drawn up by the Charity Commissioners stipulate that the Trust must retain at least a 50 per cent holding. But Ogilvie says that the trustees have taken soundings, and do not expect their plan to meet any serious obstacles.

In addition to long-term security, the sale will also provide a short-term boost to the Trust's spending on medical research. Ogilvie expects to realise about £35 million in income for each £1,000 million of Wellcome plc shares sold. If the Trust reduced its Wellcome plc holding to 25 per cent, it would have to sell more than £4,500 million worth of shares.

Peter Aldhous

CANADA

Cuts threaten Science Council

Ottawa

A STRONG voice for science may soon be stilled as part of a government effort to trim \$1,000 million from its budget.

The Science Council of Canada, which since its creation in 1966 has produced hundreds of reports on the impact of science and technology on society, is one of 46 advisory bodies that are being eliminated, consolidated, or spun off into the private sector, the country's finance minister, Don Mazankowski, announced last week. The moves are expected to save \$22 million annually.

The science council, with a staff of 29 and an annual budget of \$3.2 million, was influential in the 1970s in helping to create a national space agency to oversee the country's cooperative efforts with the United States on the space shuttle and space station. Another council report foresaw the need for a 'conservator society' that would rely on research rather than natural resources as the chief engine of development for the nation. Other major projects have examined the country's approach to science education, the need to attract more women into science and the impact on society of computer technology.

Reacting quickly to the news, the council's chairperson, Janet Halliwell, said last week that she and her colleagues want to keep the council alive with a combination

of private and government money. "Ideally, I would like to see a free-standing institute with links to universities and industry but one that also serves provincial and federal governments," she said. "Unfortunately, we don't have much time — six months if we're lucky."

In recent years, however, the council has slipped into a sort of bureaucratic limbo. In 1985 its budget and staff were cut almost in half. And its role as the premier advisory body for science policy has been challenged by the National Advisory Board for Science and Technology, created and chaired by the prime minister, Brian Mulroney.

The demise of the council is seen as a symbol of the federal government's neglect of science and technology. That neglect stands in sharp contrast to the government's repeated insistence on the importance of research and development.

When Mulroney became prime minister in 1986, for example, he promised to double government spending on science. But spending has actually declined over the past five years. Canada remains in the bottom tier of industrialized nations in the percentage of its gross national product devoted to science — half as much as its chief competitors, the US and Germany — and the figure has dropped from 1.4 per cent in 1985 to 1.3 per cent in 1985.

Both the science council and the newer national advisory board have had rough sledding of late. The board's recent recommendation to double the budgets of the research granting councils was ignored. Last year both groups, along with the NSERC and the National Research Council, told the government not to fund a \$500 million particle physics experiment, known as KAON, to be built in British Columbia and carried out with the help of the United States. But Mulroney ignored their advice and announced — timed to help a friendly provincial government in a forthcoming election — that he would provide at least \$236 million for the project.

These government policies have led to a steady decline in the level of support for the average scientist, from 53 percent of what was requested in 1983 to a current level of 46 per cent. Peter Morand, president of NSERC, has pointed out that the KAON experiment will benefit chiefly some 200 physicists, while his council's \$480 million budget must satisfy the training and research needs of some 20,000 researchers.

Although the government may feel that it has quite enough science policy advice to choose from, Stuart Smith, a past chairman of the science council, believes that the public will be the biggest loser if the council disappears. No other body in Canada, he says, can make the public so aware of science policy issues.

David Spurgeon

SOUTH AFRICA

Report asks more for academic science

Cape Town

SOUTH Africa should spend more of its research dollar on competitive, university-based research, according to a report written for the body that advises the government on science. In the face of a decline in government research spending, however, any shift in priorities would necessarily come at the expense of existing activities.

The report to the Science Advisory Council (SAC) provides, for the first time, an analysis by category of the recipients of government funding. It shows that in-house laboratories, those institutions supported by the government's five statutory councils, receive more than five times the amount of money given out to academic scientists. The 44 per cent consumed by those in-house laboratories is a far greater percentage than is the case in other industrial countries, while the 8 percent spent on academic science is far smaller than the norm. The largest single allocation, however — some 48 per cent — goes to the category of general university funding, which includes money from the state to pay faculty salaries.

Predictably, the report has been wel-

comed by the universities. Dave Woods, Deputy Vice-Chancellor (Research) at the University of Cape Town, agrees that academic research is more competitive, easier to target and more flexible than research performed in-house by the councils. But Brian Clark, president of the Council for Scientific and Industrial Research (CSIR), disagrees. "Competition for funds goes on within a research council," he says, "but at a different level".

The report to SAC was written last fall, but made public only recently. It was prepared by the Foundation for Research Development (FRD), the statutory council responsible for funding scientific research in higher education. Its conclusion is that the current structure of research support in the country "underemphasizes competitive and targeted research funding mechanisms."

According to Chris Garbers, the chairman of SAC, the report is too late to influence the council's recommendations on the allocation of the science vote for the current fiscal year. But he predicts that "it will have repercussions" next year. Already under review is the current system by which

the government makes grants available to the statutory councils and universities but does not dictate how they are spent.

The report was released at the same time as a biennial survey of R&D expenditures. The survey found a continuing drop in research and development spending, from 0.93 per cent of the country's gross national product in 1985-86 to 0.86 per cent in 1989-90. By comparison, the United States, Japan and Germany spend more than 2.5 per cent of their GNPs, and most European nations spend between 1 and 2 per cent.

One potential loser, if the foundation's report were to be followed, is the CSIR. It receives the largest slice of the science budget, but it does not fund university research. But Clark defends its role. "Ours is mission-oriented research," he says, "directed at problem-solving".

The report does not examine defence spending, which is believed to constitute as much as half of the country's overall research budget. While military spending is expected to decline in the years ahead, it's unlikely that any savings will be redirected into civilian research. **Michael Cherry**

Action on academic pay

SIR — Paramount among the perceived managerial inadequacies of Britain's vice-chancellors and principals has been their failure, as leaders, to maintain the morale and retain the respect of their most important corporate asset — the academic teaching and research staff without whom a university does not exist. As a scientist, I feel qualified to comment only on the science-based disciplines, although I am sure that my arts colleagues would wish to argue a similar case. Science-based academic staff have two main functions in a modern technology-dependent society: (1) to teach and train tomorrow's innovators, engineers, industrial scientists, doctors, surgeons and the rest to the very highest levels and (2) to carry out fundamental new and often patentable research in competition with the best scientists from other countries. Both functions are recognized by most countries to be of unquestionable importance to the future economic and cultural wellbeing of nations. Britain's university academics can be proud of their record of producing first-class graduates and PhD-level scientists, engineers and doctors. Indeed, our products are usually in advance of foreign competition not only with respect to the standard reached, but also achieving that standard (BSc at 21 or PhD at 24) a year or more earlier than their counterparts elsewhere.

What has been the reward for the teaching and research staff of Britain's universities for this dedication and productivity and for the maintenance of the highest quality of advanced education and research over the past decade? In its publication *The State of the Universities* (1991), the Committee of Vice-Chancellors and Principals (CVCP) admits that the salaries of its academic staff have fallen over a ten-year period relative to comparable groups of workers by some 25–30 per cent. It does not make mention of the fact that it has itself been setting priorities and limiting those salary rates over the same period. This is only the UK position, and comparison with international colleagues shows UK university teachers and researchers to be at least 30 per cent worse off, depending on grade, in real terms. For those of us who, despite the obvious attractions elsewhere, would prefer to serve our own country's benefit, we must protest at this shabby treatment of public servants. This position has been 'achieved' by the CVCP limiting pay increases to academic and related staff, often to several percentage points below the rate of inflation, year on year over that period. The CVCP is thus failing to provide a work environment that would

enable young scientists and engineers to conclude that there is any value in sacrificing three, six or even nine years of their lives to training for a worthwhile professional career at the end of that period. The highly predictable effects are now being felt with a vengeance. Young, high quality UK staff and research students are increasingly difficult or in some cases impossible to recruit, and the morale and enthusiasm of those already in post is at rock bottom. In addition, young research staff at the postdoctoral level (24–28 years old) see few tenure prospects beyond their fixed-term contracts, the only certainty of which is a guaranteed termination of contract usually after one, two or at most three years. High-quality takers for these positions, the seedcorn of future industrial as well as academic research and innovation, are becoming few and far between.

The potential and indeed actual acceleration of the brain-drain of economically important assets should be obvious, but not, it would appear, to our leaders, nor indeed to the government. Despite the obvious dangers, the vice-chancellors and principals have contrived year after year to hold down academic salaries as the simplest way of 'managing' the universities and complying with decreasing government funding. The CVCP says it has insufficient funds both to make reasonable pay awards and to fund the infrastructure, building maintenance, expansion and so on. The government says the universities are not underfunded. Someone is clearly not telling the truth.

Much of our problem appears to be due to the reluctance of the CVCP to argue the case vigorously for fair rewards for Britain's academic teachers and researchers, both with the public and with government. One suspects that all the publicity and impact gained by the CVCP over the past ten years to show the plight of the universities would probably not match in total the few days of publicity surrounding the recent 'defection' to the United States of the Alzheimer's research team from Imperial Medical School (St Mary's).

The CVCP is failing in its duties to key staff, and in so doing is destroying the whole ethos of advanced teaching and research as a career that young people will be interested in joining. It has now well demonstrated and even acknowledged its inability, for whatever reason, to maintain let alone increase the standard of living of university teachers and researchers, even compared to teachers at the much less advanced school level. The time is well overdue for the CVCP to stand down from the

pay negotiating process, and to call for an independent pay review body for university academic and related staff — a body that the government has already granted to the school-teaching profession. At this time of year, the university pay negotiations should be well advanced, with a settlement due on 1 April, but previous experience suggests that this will not happen. A more likely scenario is protracted 'negotiations', a request for more funding from government, followed by a pay award several per cent below the rate of inflation, paid six to nine months late. A best guess on the turn of events is that rather than attempting to redress the balance of decline over the past decade, it will currently be considering whether it can secure a figure of 2–3 per cent for this year's pay award. There are additional small merit awards for a few, and I have been the fortunate recipient on occasion, but the vast majority of highly qualified worthy individuals do not normally receive these. It is these people who deserve a fair deal, and in view of the school teachers' basic award of 7.5 per cent, payable from 1 April, there can be absolutely no justification for anything less as a basic award for university teachers also.

I respectfully suggest that the CVCP should withdraw from pay negotiation activities immediately, calling as it does so for an Independent Pay Review Panel. Time is of the essence if the rot is to be stopped before it does more harm and costs the government very real money to correct. This action by the CVCP would initiate the long haul back to a fair and worthwhile academic teaching and research career structure for our young scientists and engineers, something that is of crucial importance to the future economy of this country.

HOWARD R. MORRIS

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Power and money

SIR — I read with interest the letter by my Italian colleagues (*Nature* 355, 290; 1992) who inform your readers of their grant of L35,000 million from what they call the Italian Electric Power Authority to study air pollution and health.

Like nearly everybody else in academic life, we are short of money and have been told by our administration that we will be asked to pay part of the electricity bill out of our research money. Electric power in Italy is very expensive: now I understand why.

MARCO FRACCARO

Biologia e Genetica Medica,
Università di Pavia, Italy

Will sea levels rise or fall?

Stephen H. Schneider

Recent estimates of a global sea-level fall as a result of greenhouse warming have been uncritically accepted. A closer examination of the available data could lead to the opposite conclusion.

ALTHOUGH one cannot lightly dismiss the possibility that an increase in greenhouse gases in the atmosphere could lead to ice-sheet growth and a concomitant fall in sea level of up to 7 millimetres per year, I fear that Miller and de Vernal's conclusion, lightly hedged though it was¹, could be heavily misinterpreted. The conditions giving rise to the possibility, although plausible, are by no means assured; in fact, the opposite conclusion may be just as difficult to dismiss lightly. Miller and de Vernal suggest, for example, that equilibrium general circulation models (GCMs) that double the pre-industrial CO₂ concentration predict the greatest warming in polar regions in the winter with only "negligible (2 °C or less)" summer temperature rises. Furthermore, they note that summer solar radiation at the top of the atmosphere has been decreasing since its interglacial maximum 9,000 years ago. In other words, Miller and de Vernal argue that a combination of equilibrium GCM results for seasonal, regional temperature change in the Arctic, combined with the astronomical realignment of the Earth's orbit that gives rise to an effective alteration in the amplitude of the seasonal cycle of solar variation (the so-called Milankovich mechanism²), will increase the likelihood of greater winter snowfall and decrease that of sufficient summer snow melt; thus they predict a build-up of Arctic basin land ice and a consequent decrease in sea level with global warming. I would like to put some important mitigating perspective on these arguments.

First, with regard to orbital element variations, the timescale for changes in the summer-to-winter insolation of 1 per cent or more is thousands of years. In other words, there will be a truly negligible change in insolation at all latitudes and seasons in the next millenium relative to the past millenium. Indeed, over several thousand years there will be a trend toward non-negligible decreases in summer insolation in high northern latitudes, but this will be irrelevant on the timescale of global change, which is 100 years or so.

Another condition cited by Miller and de Vernal is that equilibrium CO₂ doubling experiments warm the Arctic region more in winter than summer. The primary explanation for this in most GCMs is the melting or thinning of sea ice, itself a

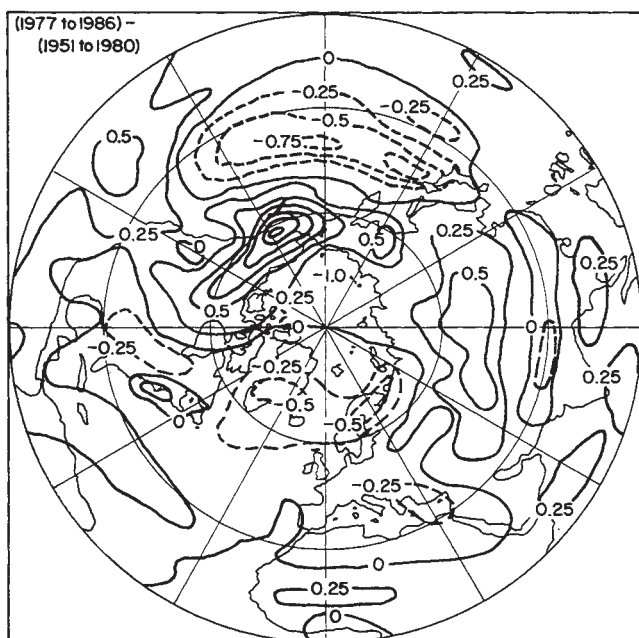
result that depends on a crude parameterization (usually a thermodynamic formulation with no ice transport included) of the sub-grid scale formation and melting of sea ice³. But even if it is true that at equilibrium the sea-ice parameterizations of GCMs provide reasonable results (a likely possibility, I suspect), the actual global change 'experiment' the Earth is now undergoing in response to the build-up of greenhouse gases is anything but an equilibrium experiment. In reality, of course, infrared radiation heat trapping from greenhouse gases is responsible for several extra watts per m² of surface heating over several decades. However, as is well known, the thermal response time of the atmosphere and land surface to such radiation forcing is months, the response time of the atmosphere and tropical mixed layers of the oceans is up to decades, and the response time of the atmosphere and deeply mixed polar waters is up to a few centuries. Some time ago, Thompson and I noted⁴ that as a result of these different response times (and the fact that greenhouse gas forcing timescales are also decades⁵), transient, regional climate changes will probably be of a different character than equilibrium results — that is, results in which all these disparate components of the atmosphere/ocean systems have had adequate time (greater than a few centuries) to come into equilibrium with the extra radiative forcing. In other words, during the global change transient, high-latitude oceans may initially warm up at slower rates than tropical oceans⁵, something that appears to be at least fairly plausible based on several preliminary coupled atmosphere/ocean GCMs^{6,7}.

As for the reliability of GCMs, despite all the caveats about their parameterizations, most assessments⁸⁻¹⁰ conclude that although it is likely that global average equilibrium surface temperature increase from a doubling of CO₂ will be somewhere between 1.5 and 4.5 °C. All assessments agree that very little can be reliably stated about the regional anomalies in climate that would undoubtedly accompany global change of such a large magnitude as a few degrees celsius. But it is the regional details of equilibrium CO₂ doubling experiments upon which Miller and de Vernal primarily base their suggestion that sea levels may fall with global warming, and because such re-

gional, transient projections are known to be unreliable, so too is any confident conclusion about whether global average warming will lower sea level.

Zwally has presented¹¹ results from satellite radar altimetry on southern Greenland, concluding that the elevation of the central part of the southern Greenland ice dome increased during 1978-86. But he added the caveat that the technique is unable to reveal much about the northern half of Greenland or the southern, lower-elevation flanks where melting takes place. Meier¹² cited Zwally's analysis, which suggests Greenland's thickening was equivalent to a figure of 0.45 ± 0.25 mm per year of sea-level lowering. Meier concluded that "this figure is not a long-term average, includes extrapolation over more than half of the ice sheet that lies beyond the satellite coverage, and incorporates little information on the marginal zone of high melt rates."

A dilemma develops, as Zwally and Meier both note, in that recent observations¹³ suggest an average rate of sea-level rise during the past 50 years or so at 2.4 ± 0.9 mm per year. I have no quarrel with the carefully hedged analyses of either Zwally or Meier. But I am concerned about the interpretation of a decrease in sea level with temperature of the presumed Greenland ice growth that Zwally associates with global warming from increased greenhouse gases. Meier suggests that new data, including these Greenland figures, justify a reduction from previous estimated sea-level changes to the year 2050. This is based on both a downwards revision of the expected contribution of small glaciers and ice caps and an additional reduction in the potential rise in sea level from a future net ice-volume change in Greenland. I question the latter conclusion, at least if it is based on Zwally's hypothesis. Zwally notes the well-known thermodynamic property of water, which is to increase its evaporation rate with temperature. Thus, it is logical to postulate an increase in global precipitation if oceans warmed, as one would expect they might in a world substantially warmed by greenhouse gases. Indeed, all computer models agree on this point, which is repeated in ref. 1. On the other hand, a warmer world would, presumably, melt more snow when local temperatures were warm enough, some-



a, Change in surface temperatures (degree C) from a linear trend fit from 1947–86 over the Northern Hemisphere (from ref. 15). b, Decadally averaged fields of surface temperatures for the 1977–86 period compared to the 1951–80 climatic 'norm' (P. D. Jones, personal communication).

thing that would be unlikely in Antarctica but would occur in the flanks and southern margins of Greenland—precisely the parts of that continent Zwally admits are not well resolved by the radar elevation technique.

But more problematic than the uncertainties in remote-sensing data is Zwally's main conclusion that "Greenland ice sheet growth is consistent with the generally warmer temperatures experienced this century. If climate continues to warm, enhanced precipitation in polar regions may offset increases in melting." This statement has been widely quoted, particularly by those who are critical of policy responses to concern about global warming, which is why I think it is necessary to add a strong element of caution to Zwally's interpretation, and indeed to its reinforcement by Miller and de Vernal.

As Zwally notes, the global average temperatures have warmed this century by around $0.5 \pm 0.2^\circ\text{C}$ (ref. 14). The problem is that annually averaged, regional-scale, decade-long temperature trends of as much as $\pm 1\text{--}2^\circ\text{C}$ occur routinely on Earth. This 'climate noise' is not related to global greenhouse gas levels, presumably, but rather is a result of a complicated set of regional-scale internal and external factors which will continue to cause such climatic fluctuations. The figure shows regional, annual surface-temperature trend fluctuations for the Northern Hemisphere between 1947 and 1986. One can say little about hemispheric or global average temperature trends of the order of 0.4°C merely by looking at specific regions as large as a continent. The United States, as is often pointed out, did not warm up appreciably unless Alaska is included (as that region warmed up much more than the global average). Although north cen-

tral Africa warmed up somewhat, north central Asia warmed up substantially more than the global average, whereas the Pacific and Atlantic regions were cooling during this 40-year period of average global temperature increase. Because Greenland is embedded in the Atlantic Ocean, it is not primarily the global average temperature (to which Zwally refers) that is important in determining relationships between temperature, evaporation, melting and net ice accumulation on Greenland, but rather the Atlantic sector temperature. The annual temperature in the Greenland area, as the figure shows, has been going down on average over the past 40 years. And as the figure also shows, during 1977–86 (the period of Zwally's radar analysis) it has dropped by about 0.5°C .

Therefore, if the North Atlantic sector annual average temperatures are used, rather than the global annual average temperatures cited by Zwally, then the opposite conclusion is reached—that is, that cooling increases the height of the ice sheet and, presumably, local warming would decrease it. I do not believe that Greenland ice volume has been measured well enough, nor temperatures or precipitation monitored perfectly enough, to draw reliable, quantitative

conclusions about how the Greenland ice sheet would change volume with any specific model of transient global warming. That caution needs to be stated ever more strongly because, as I mentioned above, some models suggest the North Atlantic may respond to greenhouse-gas forcing quite differently from the rest of the world. For example, storm tracks may be diverted in ways not forecastable based on past statistics, equilibrium GCMs or palaeoclimates, and the rate of greenhouse forcing may be sufficiently large that it could cause unprecedented alterations to circulation systems, particularly in the transient mode.

Further, it is likely to be summer temperatures, rather than mean annual temperature trends, that are most important for ice-volume/temperature trend relationships in Greenland. Therefore, before any conclusions on empirically based trends in temperature/ice-volume trends can be drawn, seasonal data would also have to be analysed. I am not aware of any such comprehensive data for the North Atlantic sector and Greenland for 1977–86.

Therefore, although satellite data such as those presented by Zwally, and the historical associations discussed by Miller and de Vernal, are to be welcomed as helping to document some of the potential mechanisms of global change, it is premature to conclude from either of these exercises that there is anything quantitative to be said about the potential relationship between Arctic sector ice-volume change and global temperature change. Moreover, if anything is suggested by the annual North Atlantic temperature drop and the southern Greenland ice dome build-up from 1977–86, it is a conclusion opposite to Zwally's—namely that ice built up when regional temperatures went down. Finally, I wonder whether the substantial recent downwards revision in global average estimated future sea-level change from global warming may itself have been premature—perhaps this too should be re-revised. □

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New dimension for Mendeleev

After more than a century, what can there be to say about the Periodic Table that is new? Merely to ask a question of such disarming simplicity, of course, signals that somebody, indeed, has something new to say.

EVERYBODY remembers the first occasion, usually in a school classroom, when Mendeleev's Periodic Table first presented itself as one of the great organizing principles of nature. The idea that all 92 natural elements might be fitted into a simple diagram on a sheet of paper must for many people have seemed the quickest way of holding the world's secrets in the head.

To be told, usually later, that the good sense of Mendeleev's conjecture about the similarities between the different elements (first made in the year *Nature* was founded) had been confirmed by everything since learned from quantum theory could only have served to confirm Mendeleev's reputation as one of almost magical insight. And it is, after all, not just a legend, but the truth, that he predicted that there would be found the element now called scandium.

Bohr was one of the first to make it clear that, by the 1920s, even his version of the quantum theory accounted for the regularities, and made plausible some of the irregularities, of the Periodic Table. On the onion model of atomic structure, in each successive completed shell of electrons there are $2n^2$ electrons, where n is an integer greater than or equal to unity and representing the order of occurrence of the electron shell in the onion model. Even young people can be entranced to know that the factor "2" arises simply because there are two allowed directions of electron spin.

But which version of the table should be kept in mind? Classroom walls are hung with two — a more or less square array of symbols (with hydrogen and helium displayed separately at the top) and another shaped like a schematic drawing of an Aztec or Babylonian temple, consisting of several superimposed layers with the broadest at the bottom. The two versions differ simply in their arrangements to accommodate elements such as the rare earths, but the result must be to leave many with the impression that Mendeleev had not made up his mind about something of importance.

Luckily, as we all now know, there is no hidden scandal. In adding electrons successively to an empty shell in an onion model of an atom, the first eight electrons go where they are expected to fit, in the s and p states (holding two and six respectively), but then it proves to be less energetically favourable to accommodate succeeding electrons in the same shell, into what should be the d state, with a capacity

for ten. At least two of them slot more easily into the next higher shell, whereupon the unfilled states in the next innermost shell can be filled (which process accounts for the transition elements from scandium to nickel inclusive).

The implication is that the eight-fold periodicity in the Periodic Table (and about the only true periodicity there is in it), represented by the elements from lithium to neon inclusive, and from neon to argon, is strictly spurious in origin. With elements whose atomic number is greater, there are inevitably more complicated transitions, as with lanthanum, the first of the rare earths, where, with the $6s$ state as well as the $5s$ and $5p$ states filled, the electron added to the full complement of barium's electrons goes into the empty $5d$ state rather than into the still empty $4f$ state.

It is of some interest that this curious behaviour, and the two series of transition elements as well as the rare earths and the actinides (mostly artificial elements), owe their existence to the present value of the electron charge. If it were very different, even Mendeleev might not have been able to spot regularities among the data with which he was confronted.

Many of the irregularities are more particular and more perplexing. Everybody can accept that elements such as helium and argon have much in common, even if the collective term "inert gases" has recently been rendered a misnomer, but what does carbon have in common with tin and lead, all of them members of what are called the Group IV elements in the table? More generally, why in any group of elements with supposed affinity are those with greater atomic number more inclined to form cations as if they were metals?

These are some of the questions now taken up by Leland C. Allen of Princeton University (*J. Amer. Chem. Soc.* **114**, 1,310; 1992) and answered with the simple declaration that it is "clear that something is missing". The Periodic Table, he says, cannot be complete as a two-dimensional array, whatever its shape. He says there must be a missing dimension, which he argues must somehow be a function of the electronic energy of the atoms concerned. He calls the missing dimension "configuration energy" and proceeds to define it, at least formally, as the average one-electron valence shell energy of a ground-state free atom.

Nobody will blame Mendeleev for not having thought of that. Moreover, con-

figuration energy as defined can in principle be measured by spectroscopists. It is only necessary to measure the ionization potentials of the valence electrons (which may differ in the s , p and d states) and then to take an average weighted by the number of the electrons in the different states. But, like all empirically conceived measures — Allen refers to Pauling electronegativity index, dating from 1932, as another — what matters is whether configuration energy resolves enough of the inconsistencies in the Periodic Table to win the hearts and minds of men with an interest in these matters.

What Allen is most pleased with is that configuration energy as defined rationalises what he calls the metalloid band in the table, which is the diagonal line in the rectangular representation of the table reaching from boron (element 5) to antimony (element 51). All the elements to the left are metals and have small configuration energy, all those to the right are non-metals with higher configuration energy — and those at the boundary have virtually the same configuration energy as each other.

Allen argues that his index, which he says subsumes Pauling's, "uniquely qualifies" as the extra dimension missing from Mendeleev's table. On the evidence presented, that may well be the case, although it will be interesting to see whether it can make predictions about, say, the properties of simple compounds that have not yet been made which are arresting enough to compel general attention.

A more obvious difficulty is that the extra dimension may be half a century too late. At the beginning, the Periodic Table was most spectacularly useful as a predictive tool, but by 1932 it had become mostly a mnemonic. Since then, two generations have learned to rationalise some of the irregularities of the Periodic Table by arm-waving of a pseudo-theoretical character. That the inert gases are more or less chemically inert is justified by the extra binding energy of electrons in a complete valence shell of electrons whose orbits are symmetrically hybridized with each other, which means that excitation to the next higher energy level (as required in some virtual sense, at least, to form a chemical bond) is relatively difficult. Those who use such arguments do not, of course, claim to have made the calculations, but that is no greater shame now than some of the other arguments of the same kind used in the past century.

John Maddox

The worm turns and delivers

Peter Little

Supporters of large DNA sequencing projects will take heart (and find much to learn) from the report by J. Sulston and colleagues that appears on page 37 of this issue¹. Sulston *et al.* describe the first results of the *Caenorhabditis elegans* genome sequencing project, and have come up not only with hitherto unknown genes but also with fresh and biologically relevant information.

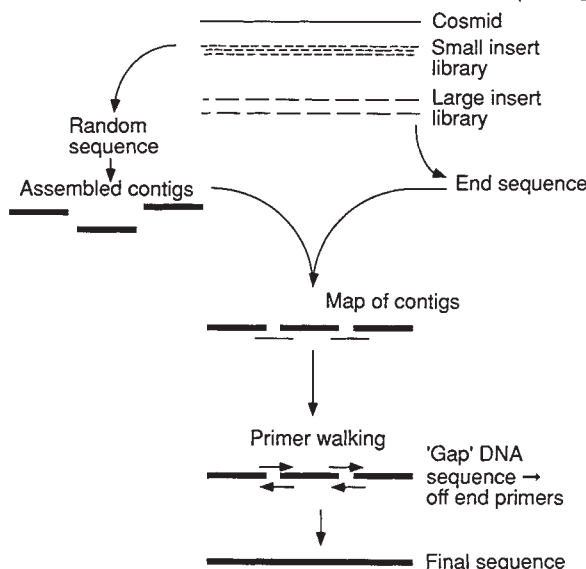
Detractors of sequencing projects contend that biological knowledge comes more effectively and more cheaply from conventional studies of the behaviour of genes, mutations and molecules; the discovery of genes for which functions have not been found, they argue, is of no more consequence than random cataloguing. Yet the new work has picked up significant numbers of genes of known function that have not previously been identified. It demonstrates that DNA sequencing is not only a matter of cataloguing but also expands the biology of existing protein molecules.

The group concerned, a collaboration between two laboratories, has established the sequence of 121,298 base pairs (bp) of DNA cloned in three cosmids isolated from the *C. elegans* chromosome III (*C. elegans* is a small nematode worm that has long been the subject of biological investigation). This region of the genome was chosen because it is well covered by cosmid and yeast artificial chromosome DNA clones², and because classical genetic analysis had shown it to be rich in genes. The technical approach Sulston *et al.* used to generate the sequence is an indicator for future studies on large genomes: the outline of the methodology is given in the figure.

Two types of library are constructed from each cosmid: 1–3-kilobase (kb) DNA fragments shotgun-cloned into vectors capable of producing single-stranded templates (small insert libraries) and, separately, 6–9-kb fragments (large insert libraries). Between 102 and 360 small library clones from each cosmid are sequenced using fluorescent labels and automatic sequencers, with an average of 390 bp read. Software is used to edit the raw trace data, which are then stored, the DNA sequence being extracted and assembled in sequence using Staden's assembly programs³. The result is a number of non-overlapping, continuous chunks (contigs) of DNA sequence.

At the same time, the ends of a number

of random large insert clones are sequenced and compared with the small-insert generated contigs: this generates a map of contigs linked by large insert clones, the end sequences of each large insert clone being found in adjacent contigs. Next, 100–417 fluorescent primers are generated from the ends of the contigs, which are then used to generate sequence from the appropriate large insert clones (primer walking), gen-



The strategy for sequencing the *C. elegans* genome starting from mapped cosmid clones. Thick lines represent DNA sequence, thin lines cloned DNAs. Sequence represented as an arrow is the product of primer walking.

erating the complete sequence. In total, about 170,000–180,000 bp (redundancy of 3.6–4.3) of sequence was obtained from each cosmid, generated from between 440 and 589 gel reads.

What of the vexed question of expense? Sulston *et al.* estimate it to be \$1 per base, about half of that being labour cost. But they point out that \$0.50 per base should be achievable: the important point here is that cost reduction does not demand dramatic technical advance, because through economies of scale the savings will be realized as the project goes to full production phase. Next year, the goal is sequencing 800 kb, the following year 2,000 kb.

Analysis of the sequence itself detects fairly complex duplications and multiply repeated sequences (21 copies of a 59-bp sequence in one case), making DNA sequence determination by primer walking down a cosmid almost impossible. A large stretch of tandemly repeated DNA sequence NGG was seen in one region: this is the sequence at the human X-linked fragile site.

Computer analysis with the GENE-

FINDER program suggests that 27 per cent of the DNA is coding, and that 34 genes are contained in the 120-kb region. Some of these putative genes encode proteins with functions that have already been identified, as was quickly shown to be the case by database comparisons of sequence using the BLAST software⁴. The strongest similarity was to the gene encoding the rat vacuolar proton pump: other finds were sequences very like those coding for acetyl-CoA acetyltransferase, glutathione reductase, arsenical pump-driving ATPase, the TcA transposase, a host-protective factor from the parasitic nematode *Trichostrongylus colubriformis*, and the L11 ribosomal protein. More limited similarities were to genes for adenyl cyclase, CDC25, IE110 of herpes simplex and the giant secretory protein. All this, it has to be said, does not add to our understanding of nematode biology. At least not yet.

But it is difficult to believe that the unveiling of these genes, none of which had been identified before, will not suggest new lines of work to biologists in, for example, the pump field.

Those working on the human genome project can perhaps learn another lesson. It has been argued^{5,6} that sequencing of genomic DNA is not a cost-effective way of understanding the genetic composition of complex organisms, and that sequencing of complementary DNA (which I have discussed in these pages⁷) is perhaps a better approach. The intellectual difference

is that genomic sequencing generates information about intergenic regions, thereby prompting a host of additional studies on promoter regions and DNA sequence evolution, to name but two areas. What Sulston *et al.* show is that gene density can be very high (35 genes in 120,000 bp, or a gene every 3.5 kb), although average density is probably lower. The same laboratories involved in the work described in this issue¹ have sequenced 1,517 nematode cDNA clones (J. Sulston and R. Waterston, personal communications). Statistical analysis suggests that there are 15,000 genes in *C. elegans* (1 gene per 6.5 kb), but the confidence limits on that estimate are broad (95 per cent probability, 6,500–40,000).

In higher animals, estimates of gene

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number are very loose: many of the data are based upon reassociation techniques which have technical limitations, and also upon biological assumptions that are often untested (for example, that there is no significant proportion of genes that are expressed in very tight developmental windows). The question is explicit. Given

that we do not know the gene density of any but tiny regions of vertebrate genomes, what exactly is the experimental basis of contentions that cDNA sequencing alone is the way forward? □

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HUMAN GENETICS

The costs of instability

Kay E. Davies

THE identification over the past year of the mutations involved in three neurological disorders — fragile X syndrome¹⁻³, spinal bulbar muscular atrophy⁴ and, now, myotonic dystrophy⁵⁻¹⁰ — has revealed that a common genetic mechanism underlies them all. This creates a very exciting prospect: the rapid identification of other disease genes having a similar defect, the possession of sequences of unstable DNA.

The myotonic dystrophy (DM) story has emerged very quickly (as described in News and Views last week), the elements coming from several laboratories and appearing in *Nature*⁵⁻⁷ last month, and in the latest issues of *Cell*⁸ and *Science*^{9,10}. The disease is an autosomal dominant multisystem disorder; mildly affected individuals have cataracts but little or no myopathy, whereas severe cases are characterized by muscular hypoplasia and mental retardation¹¹. An increase in severity of the disease in successive generations is observed in susceptible families (genetic anticipation). Ascertainment bias has been suggested to explain this pattern of inheritance¹², but the new work shows that anticipation is due to the progressive amplification of a DNA sequence.

The amplification of sequence was first observed using genomic DNA from DM individuals³⁻⁷, and has now been shown to be due to the expansion of a CTG repeat at the 3' end of a transcript designated DM-1 (refs 8-10). Normal individuals also show the variable numbers of the CTG repeat with a heterozygosity frequency of 75 per cent; the modal number of repeats is 5 with the largest being 77 (ref. 8). In contrast, minimally affected DM individuals have at least 52 copies of the repeat. Assuming that larger amplifications are expansions of CTG, then severely affected cases must often possess more than 1,000 copies of the CTG trinucleotide. This type of mutation readily explains the molecular basis of genetic anticipation. Asymptomatic carriers in early generations pass on the mutant chromosome to their offspring with an increase in the number of repeats. Consequently, in successive generations, the repeat length

increases and a greater severity of the disease and earlier age of onset are observed. Amplification of the repeats occurs whether the mutation is passed through the male or female line. The present data do not, however, bear upon the question of why severe congenital cases are almost exclusively maternally transmitted.

The expansion of the CTG repeat seems to have occurred only in a specific population subgroup, because there is linkage disequilibrium between DM and polymorphisms closely linked to the disorder on chromosome 19 (refs 13, 5). The initial amplification event (perhaps a doubling of the number of repeats) may have existed in populations for some time before finally expanding to give a clinical phenotype. Studies of populations as yet unaffected by the disorder will be necessary to investigate this further.

An unusual characteristic of CTG amplification is that it is also associated with somatic heterogeneity. Individuals with large amplifications show a smear of fragments rather than a discrete allele. It will be of great interest to determine whether the same multiple pattern of mutant fragments is observed in all tissues, as the phenomenon could also contribute to clinical variability.

Sequencing studies demonstrate that the CTG repeat occurs within the 3' untranslated region of a gene. The transcript is approximately 3 kilobases in length and is expressed at high levels in heart and to a lesser extent in skeletal muscle and brain^{8,9}. Database sequence searches reveal similarities with genes encoding cyclic-AMP-dependent protein kinase, the closest similarity being to protein kinase TKR-YKR from *Saccharomyces cerevisiae*. Abnormalities in function or regulation of such a molecule would fit well with the pleiotropic effects exhibited by DM patients; indeed, a defect in such a protein was predicted by Roses and Appel almost two decades ago^{14,15}.

It is not immediately apparent why a mutation at the 3' end of a gene should result in a dominant phenotype. It seems unlikely that the expression of such a

gene would be sensitive to dosage. There may be critical regulatory factors in the 3' region of the gene which are disrupted by the mutation in a way that would have a dominant effect (there are examples of such regulatory elements in the nematode^{16,17}). Detailed knowledge of the regulation of DM-1 will be required to elucidate this further.

Is the genetic mechanism underlying DM instructive for determination of the molecular basis of other genetic diseases? The fragile X syndrome is already well documented; in this case, genetic anticipation is also explained by the amplification of a trinucleotide repeat (in this instance CGG)¹⁻³. In a similar manner to DM, an increase in repeat size is seen in successive generations and somatic heterogeneity is evident. In fact, it was the nature of the fragile X mutation that prompted Fu *et al.*⁹ to scan cosmids covering the DM candidate region for trinucleotide repeats.

Trinucleotide expansion also underlies X-linked spinal bulbar muscular atrophy⁴. Here the number of CAG repeats in the androgen-receptor gene increases with either the maternal or paternal transmission 30-35 per cent of the time overall (K. H. Fischbeck, personal communication). But there is no genetic anticipation, and dramatic expansions have not been observed (patients, 39-60 repeats; controls, 13-30 repeats). This lack of large numbers of repeats may explain why somatic instability is not evident in these mutant alleles.

The three examples discussed here show that it may well be worthwhile to scan complementary DNA libraries for cDNAs carrying trinucleotide repeats. It seems likely that several other genetic disorders are caused by the instability of GC-rich trinucleotide repeat elements, a prospect that will keep human molecular geneticists busy for some time. □

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Superplumes and superchrons

Mike Fuller and Robin Weeks

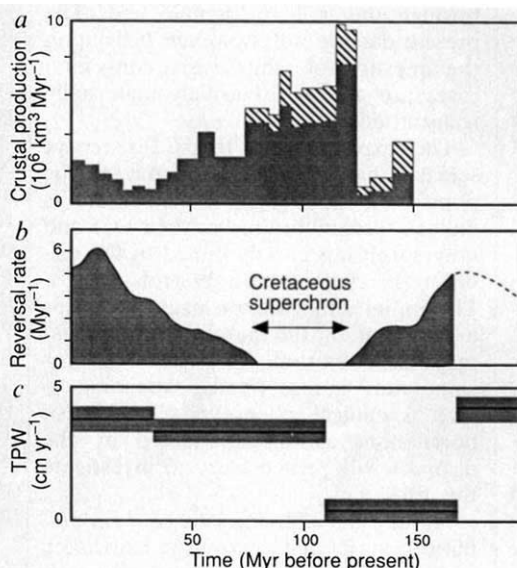
IN a bold stroke last year, Roger Larson proposed that a 'superplume' erupted in the Earth's mantle 120 million years ago, leading to unusually rapid production of oceanic crust, and perhaps stabilizing the polarity of the Earth's magnetic field¹. The latter point tied the superplume in to the Cretaceous superchron, a 40-million-year period over which the geomagnetic field remained in its normal polarity. Now Larson puts forward, with Peter Olson, a mechanism by which mantle activity can modulate circulation in the Earth's core, where the field originates, and so prevent magnetic reversals².

The study of mantle plumes is turning out to be a unifying concept in geology, bringing together people with a wide variety of interests. Plumes are ascending columns of hot mantle, thought to originate near the core-mantle boundary, which traverse the mantle and terminate in the overlying lithospheric plates as hotspots. Geologists are intrigued by plumes as an explanation of chains of volcanic islands, of oceanic plateaux and of continental flood basalts. Geochemists see them as a means of sampling the isotope chemistry of the deep mantle. Geophysicists of a seismic bent see relations between plume distribution and the new tomographic studies of the deep interior, while students of the gravity field of the Earth correlate the geoid with the distribution of hotspots, and geodynamacists seek to model plume structure. Even atmospheric scientists have joined in, because of the potentially important effects plumes may have on the atmosphere through the gases they liberate at the Earth's surface.

Larson's paper last year¹ developed another aspect of plume studies, by providing a quantitative basis for the earlier suggestion by Peter Vogt³ that there is a correlation between hotspot activity and the frequency of geomagnetic reversals. Larson argues that, for the past 150 million years, "magnetic reversal frequency correlates inversely with mantle plume activity, as measured by the volume production rate of oceanic plateaux, seamount chains and continental flood basalts". As Keith Cox underlined in these pages last year⁴, proving the correlation is not so simple. Certainly the record of geomagnetic reversal frequency is firmly established (see figure). But the estimation of plume activity, through summing the production of oceanic plateaux, seamount chains and

flood basalts, involves more uncertainty. However, it is the relative timing of the superplume and the superchron that presents the greatest difficulty and indeed David Loper⁵ suggests that the thermal inertia of the region from which the plumes originate may invalidate Larson's crucial idea that the plume activity would rapidly change the core's heat flux. Nonetheless, the idea is intriguing, especially in relation to other ideas about geomagnetic field reversals.

The essence of the model is that the



Comparison (after Cox⁴) of estimated crust production (a), geomagnetic reversal rate (b) and true polar wander (c, after Courtillot and Besse⁸). Crust production, calculated from the growth of oceanic plateaux, seamount chains and continental flood basalts, increased suddenly just as the reversal rate fell to zero, and decreased as the reversal rate picked up again, according to Larson¹. But the zero in TPW preceded the Cretaceous superchron. (Hatched shading in part a, estimate of crust production on eastern Pacific floor more than 60 Myr ago, assumed now to have been subducted.)

perturbation of the thermal boundary conditions of the outer core by the formation of superplumes increases the vigour of convection in the outer core. This would switch the Earth's dynamo from a reversing, a.c. state to a non-reversing, d.c. state. The justification for this suggestion comes from an earlier analysis of the geodynamo problem by Olson and Lee Hagee⁶. Their dynamo falls into the class of intermediate dynamos. It is not simply a kinematic dynamo, in which one chooses a particular preferred fluid flow in the outer core and, without taking account of the effect of the field on the flow, calculates the field generated (or, rather, regenerated, as circulating conducting fluids can pro-

duce magnetic fields only where one exists already). Nor is it a solution of the full set of magnetohydrodynamic equations.

The main regenerative process near the inner-core boundary is specified in the model, but the damping effect of the field on the convective process is modelled with a magnetic-energy density term. In this model, reversals are an oscillation intrinsic to the dynamo process. Calculations reveal that vigorous convection gives strong, nonreversing dipole fields, while less vigorous convection gives weaker, oscillatory dipole fields. So the mechanism required to couple plume activity to reversal frequency follows naturally from this dynamo — plumes that erupted before the superchron will have strengthened convection, giving a strong non-reversing dipole field.

Other popular models of field reversals, developed from the ideas of Allan Cox⁷ predict a correlation quite unlike that described by Larson. According to Cox's approach, a field reversal is not part of the normal dynamo process, but results from an instability or perturbation. A strong perturbation in the outer core feeds sufficient reverse flux into the dynamo to overcome the main dipole. Reversal recurrence intervals then follow Poisson statistics because, although there are many instabilities, only rarely does one result in a field reversal. Increased convective vigour should generate instabilities more frequently so that an increased reversal rate would be expected. The phase relations between initiation of plumes and changes in reversal rate would also need to be very different from those proposed by Larson and Olson, if plumes are to account for the Cretaceous superchron.

Vincent Courtillot and Jean Besse⁸ argue that increased plume eruption increases mean lithospheric plate velocities and true polar wander (TPW) about the mean pole. They observe that a period of low or insignificant TPW in the record corresponds with the decrease in reversal frequency before the superchron and that TPW is not seen again until some time during the superchron (see figure). In their scheme, the decrease in reversal rate before the Cretaceous superchron is caused by a decrease in core convection due to a build up of heat at the core-mantle boundary. The excess heat is eventually released with the eruption of plumes towards the end of the Cretaceous superchron which later generated flood basalts after the superchron. The plume initiation gives the increase in core convection, which in turn causes an increase in reversal rates.

In the face of these contradictory pre-

Viruses, cancer and the MHC

William Klitz

dictions for the effect changed convective vigour should have on reversal rate, we should see if any of these relations is accessible to experimental test. If the temperature on the core-mantle boundary changes, the flow in the outermost core must presumably change⁹. At present this flow is intimately linked to secular variation of the field¹⁰, so that if the flow were fundamentally different during the superchron, secular variation should also be different. To test Larson and Olson's suggestion, then, we should compare the secular variation then and now. Or if the flow controls features of the reversals themselves^{11,12}, there is another way of determining the ancient flow on the outer surface of the core. Reversals close to the superchron should differ from those during times of more frequent reversals, in Larson and Olson's picture. But these tests are based on data and interpretations that are themselves controversial. The difficulties are compounded by the fact that we do not even know whether the main geodynamo is deep and merely perturbed by the surface flow, or whether the surface flow is a more fundamental aspect of the dynamo.

The clearest prediction of Olson and Hagee's dynamo is the inverse correlation of field intensity and reversal rate. This is also testable palaeomagnetically. We palaeomagnetists cannot yet answer these questions definitively. Indeed each question pushes our techniques to the limit. But with the new motivation, answers can be expected soon.

It remains to be seen how successful the ideas of Larson and Olson will be in the long run. As is so often the case in geophysics, having a good idea is only half the solution. The next job is to demonstrate that it happens in the real world. A great virtue of the new model is that it makes clear and potentially testable predictions, which are already stimulating important work. □

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JUST as some intriguing plot possibilities are beginning to materialize between viral infection and mutational loss of tumour suppressor genes for some cancers¹, another actor — the major histocompatibility complex (MHC) — makes its appearance. Last year Wank and Thomssen² presented evidence for an association of squamous cell carcinoma of the cervix in humans with the MHC; now, on page 66 of this issue³, Han *et al.* report that both the regression and malignant conversion of viral papillomas in rabbits are linked to the MHC.

Loss or alteration of function through mutation is the basis on which the usual model of 'genetic disease' relies. Discovering the Holy Grail of disease genetics amounts to locating a mutated site on the genome associated with a disease, thereby pinpointing the cause of a malady. But we must, perhaps, remind ourselves that monogenic inheritance of traits does not apply for many diseases.

Genetic variation in the HLA region, for example, presents a very different pattern of association with disease (in humans, MHC loci or their products carry the prefix HLA). When HLA variation is associated with a disease (for example rheumatoid arthritis or insulin-dependent diabetes), it seems that only a small fraction of the individuals carrying a susceptibility allele will have the disease; most individuals will be quite normal and free of symptoms. This is best explained by the pivotal role of HLA variation in the specific immune response — foreign (nonself) protein antigens activate the immune system depending on the particular allelic forms of the HLA molecules expressed by an individual. In most HLA-associated diseases, altered cellular physiology, at least some of which may be virally induced, precipitates autoimmunity. An individual's own proteins are presented by one or more of an affected person's histocompatibility molecules, and recognized as nonself to activate the cellular and humoral immune responses. Unaffected individuals carrying susceptibility alleles for a disease must be missing genetic or environmental components essential for disease expression.

At this point the evidence of the association of cervical carcinoma with HLA presents us with the familiar issue of determining just where, among several highly polymorphic loci in linkage disequilibrium, the consequential contribution lies. Wank and Thomssen² emphasized the importance of HLA-DQw3 as the locus most closely associated with cervical carcinoma, and the

report⁴ of their further molecular typing of the cervical carcinoma patients, which appears, with other comments, in the Scientific Correspondence section of this issue, seems to sustain that impression.

Examination of their data, however, suggests other possibilities. The HLA phenotypes of the 94 individuals in the original study² are divisible into three categories: cervical carcinoma, cervical dysplasia and 'other' conditions. Cervical dysplasia is a precursor state to cervical carcinoma. The DQw locus allele frequencies of cervical carcinoma and cervical dysplasia are statistically indistinguishable, but these two categories are quite different from both the 'other' group and outside controls. The DQ differences are due to an increase in DQw3 and a decrease in DQw4. The DR frequencies of the cervical carcinoma and cervical dysplasia groups are even more distinct from the control and 'other' groups, with DR5 in excess and DR4 and DR6 reduced. As Helland *et al.* note for their Norwegian data (see Scientific Correspondence⁵), because of disequilibrium with both DR4 and DR5 (which have opposite effects on susceptibility), DQw3, as defined, is unlikely to be the cause of the association. Subdivision of DR5 into its five recognized alleles by DNA typing might result in even stronger effects.

It is not too unusual that, when an HLA-disease association is published, subsequent studies may not confirm the original report. Indeed, in their contribution to Scientific Correspondence⁶, Glew *et al.* report finding no association between cervical carcinoma and class II HLA in their sample of patients in northwestern England. Besides typing quality (of which more below) and type I statistical errors, two biological phenomena add to this problematical situation. One is disease heterogeneity, in which the same phenotypic result is achieved by different routes; HLA associations, for example, are clearly involved in differentiating between the forms of diabetes — only insulin-dependent diabetes has striking HLA associations. A second source of the difficulty in replicating HLA-disease associations is HLA differentiation among populations. The microheterogeneity in HLA frequencies in European populations, for example, creates the possibility of linkage disequilibrium between disease and HLA in one population but not in another. This possibility rests on the strength of the association: the presence of ethnic differentiation in HLA frequencies among, say, a com-

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bined group of individuals with Irish, French and Polish backgrounds sampled in California, is unlikely to modify the conclusions of HLA effects in the face of strong disease associations.

Achievement of the twin goals of measurement in any science (that is, accuracy and precision) is now in sight with the advent of DNA typing of HLA class II alleles (DNA typing systems for the class I loci are under development). In one comparison of serological and DNA typing for the DR locus (DRB1), 11 per cent of serological typings failed outright and 25 per cent of the rest contained typing errors, compared to the DNA-based standard⁷. Moreover, on the question of precision, the DR4 serological category is, for example, now known to be composed of eleven DRB1 alleles, none of which is detectable by serology. It is time to stop the foot-dragging, and make the overdue move of adopting DNA typing instead of serotyping for class II alleles.

The work of Han *et al.*, which involved New Zealand rabbits infected with rabbit papilloma virus³, may well turn out to provide a beautiful experimental model for examining the virus-MHC connection in malignant conversion. We would like to know more of rabbit MHC genetics: how many class II loci are expressed, and the allelic variation and the mapped locations of each, and the haplotypes and the location and variability of other candidate genes including the tumour necrosis factor and peptide transporter loci. All of this is surely coming, but for now we note that MHC allelic effects occur both as susceptibility and protective influences on the viral effect on epidermal tissue.

Are the links between human papilloma virus (HPV) and HLA of especial note? If HPV is like a proposed way in which the human immunodeficiency virus relates to HLA, then infectivity may not be the issue; rather, it may be the transition to actual disease, whether cervical carcinoma or AIDS. Is the HPV havoc within a precancerous cell exposing 'new' self-antigens visible to all but those individuals bearing susceptibility alleles, or is the association more direct with HPV infection tolerated and per-

mitted only in the presence of appropriate HLA products? Note that the viral status of the patients is given in none of the reports described here. Do HPV types (and dozens are recognized) influence the HLA associations with cervical carcinoma, and, further, do the other anogenital cancers of HPV also have HLA associations?

Further pointers to a viral-MHC connection with neoplasia are seen in nasopharyngeal carcinoma, which has been linked to both the Epstein-Barr virus (EBV)⁸ and genetic variation in the HLA region⁹. Another cancer, Hodgkin's disease, which was one of the first diseases for which an HLA association was reported¹⁰, is also associated with

MAGNETIC STORMS

Tweaking the magnetosphere

W. S. Kurth

THE dramatic effects of magnetic storms above the Earth's neutral atmosphere have been highlighted several times during the recent maximum in the cycle of solar activity¹. But although the loose association between solar activity and the terrestrial storms has long been made, the details of cause and effect are

EBV¹¹. Data reported at the International Histocompatibility Workshop in Yokohama last November implicate a weak but consistent association with the DPB1 locus. Work currently underway, the aim of which is to type the rest of the class II loci, promises to pick up stronger signals from this region.

We await the results of a suite of experiments on the links between the immune system, viral infection, and neoplasia. They promise to increase both our biological understanding and, in the long term, our well being. □

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FIG. 1 The Northern Lights (aurorae) can move far south during a magnetic storm.

not clear. From an analysis of the circumstances surrounding five of the most intense storms of the previous solar cycle, Tsurutani *et al.*² suggest that extreme southward magnetic fields in and in front of flare-associated coronal mass ejections that intercept the Earth's magnetosphere are most likely to generate the largest events.

When there is little solar activity, the quiescent solar wind draws the Earth's magnetosphere out into a long tear-drop shape as it moves by. Minor variations in the wind speed or solar magnetic field direction result in minor fluctuations of the magnetosphere and drive nearly continuous auroral displays at high latitudes. Major disturbances in the solar wind, however, can severely distort the con-

figuration of the magnetosphere, even to the extent of pinching off part of its long tail resulting in a relaxation of the stretched field lines and the subsequent dumping of energy stored there in the form of spectacular aurorae (Fig. 1) moving to abnormally low latitudes. Brilliant auroral displays sometimes seen

as far south as Texas are the silver lining to the storms. But the 'cloud' itself includes extensive power failures, disruption of electronic communications and undermining of navigation systems. Understanding how such storms arise and learning how to foresee them is a matter of more than academic interest.

Tsurutani *et al.* are the most recent of a series of groups attempting to identify the most effective features of flare-associated disturbances in generating storms. Gosling and colleagues^{3,4} have demonstrated that a large majority of severe storms are related to the passage of a coronal mass ejection from the Sun accompanied by an interplanetary shock wave, an indication that the solar plasma is moving significantly faster than the undisturbed solar wind ahead of the disturbance (Fig. 2). In fact, for the population of storms studied, it seemed that the initial speed of the mass ejection close to the Sun might be the most important factor determining its effectiveness in generating a storm.

Tsurutani *et al.* studied only the five most intense magnetic storms of the previous solar cycle between 1971 and 1986, as indicated by the magnitude of the change in the equatorial magnetic

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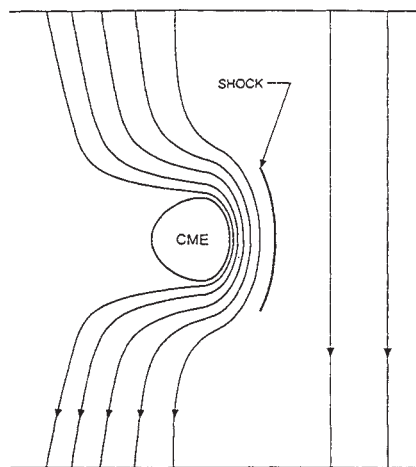


FIG. 2. Diagram of a coronal mass ejection (CME) moving from left to right which shows the compression and draping of solar magnetic fields in a southward-directed configuration ahead of it. (From ref. 2.)

field. They contend that each of these storms was caused by generally simple coronal mass ejections which had extensive southward magnetic fields either preceding the driving gas or in the driving gas itself. A southward component of the interplanetary magnetic field has long been known to be important in the stimulation of geomagnetic activity, presumably because this orientation would seem to assist the merging or reconnection of field lines between the interplanetary and geomagnetic fields. Each of the five largest magnetic storms of the last solar cycle was associated with the passage of coronal mass ejections and associated interplanetary shocks and had significant regions of southward fields. The authors argue that the strengthened southern-directed field is often a natural consequence of the way the magnetic fields in front of an advancing disturbance are swept up in front of or draped around the fast-moving gas.

The greater emphasis on the role of southward magnetic fields in the new work, compared with the earlier studies by Gosling *et al.*, is probably a result of the selection by Tsurutani *et al.* of just the largest storms. Both teams admit that the coronal mass ejections often do not lead to significant magnetic storms whether accompanied by a shock or southward magnetic fields. A unique set of necessary and sufficient conditions for a major magnetic storm has yet to be identified. □

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Tunable polymer diodes

David Bloor

DURING the past three years there has been rapid progress in the development of electronic devices based on conjugated, semiconductive polymers. Initial studies of hybrid field-effect transistors (FETs) formed from polyacetylene films coated on silicon substrates were soon followed by the development of flexible, totally organic transistors with reasonable performance (see my earlier News and Views articles^{1,2}). At the same time reports were appearing of light-emitting diodes (LEDs) based on polymers³. On page 47 of this issue³, Burn *et al.* describe block copolymers made of alternating conjugated and nonconjugated sequences, which mimic the electronic structure of group-III-group-V compound semiconductor quantum wells⁴. In giving control over the frequency of light from the polymer and in increasing the emission efficiency, the alternation marks a significant further development in this field.

A well known model system used to illustrate elementary discussions of quantum theory is the 'particle in a box' (Fig. 1a). An electron free to move along a line between two infinitely high energy barriers has quantized energy levels. The allowed energy values are proportional to the square of an integer quantum number which takes values

from one upwards. These energies are also inversely dependent on the square of the distance between the potential barriers. Thus, if the box is large the spacing of energy levels is small and the distribution approaches a continuum. If the box is small the energy levels are well separated. This simple model has been used to describe the behaviour of electrons in molecules (the 'free-electron' model⁵) and in semiconductor quantum wells.

The simplest linear conjugated molecules, the polyenes, consist of (CH=CH) repeat units. Each carbon atom contributes one free electron; these π -electrons are represented formally as the second bond in the double bond, but are regarded as free to move throughout the molecule. The two free-electrons per (CH=CH) repeat unit fill the lowest empty energy level of the 'particle in the box' model. Optical absorption occurs when an electron is excited from the highest occupied level to the lowest empty level. The energy at which the absorption is found is predicted to be inversely proportional to the number of (CH=CH) units in the molecule. For an infinitely long chain the predicted energy gap is zero, so that it would have the properties of a metal.

This result does not accord with either the observed optical absorption energies of short polyenes⁶ or the fact that polyacetylene is a semiconductor⁷. The simple free-electron model has a constant potential at the bottom of the well. A more realistic model for a molecule uses a modulated potential (Fig. 1b) which reflects the presence of the atomic nuclei in the molecule⁸. This, and other more sophisticated models⁹, predict a finite energy gap for an infinite chain but retain a strong dependence of absorption energy on molecular size for small molecules. It is this fact that explains the blue shift in emission seen by Burn *et al.*, as they reduced the mean length of the conjugated blocks interspersed between insulating, nonconjugated sequences in the copolymers they describe in this issue³ (Fig. 2). The shorter sequences also give greater radiative efficiency, because they give fewer alternative pathways for nonradiative removal of energy.

The precise control of the epitaxial growth of thin films of compound semiconductors possi-

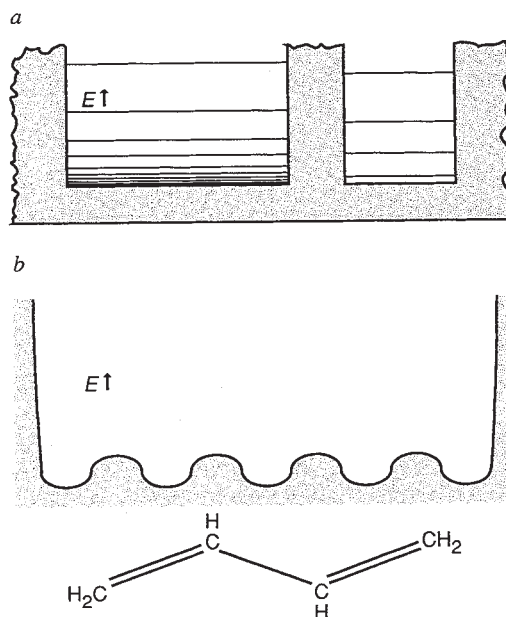


FIG. 1 a, 'Particle in a box' model of an electron in a molecule or quantum well. The electron is free to move between the walls (representing infinite potential barriers) but not through them. It can take only specific quantized energy levels, which are further apart in smaller confines. b, A more realistic model for a polyene, showing the effects on the potential introduced by the molecule's atomic structure.

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ble with the methods of molecular beam epitaxy and metal-organic vapour-phase epitaxy enables electronic engineers to build up quantum-well structures. A typical structure is made from alternating layers of gallium arsenide (GaAs) and aluminium arsenide (AlAs). The two materials have different band gaps and electrons are confined to the layer

ever, are amorphous and the conjugated blocks vary in both size and separation and are randomly distributed in the polymer film. The behaviour of idealized polymeric quantum-well structures has been explored theoretically^{10,11}. However, the synthesis of polymers with precise block size and an ordered, chain-extended structure has not yet been

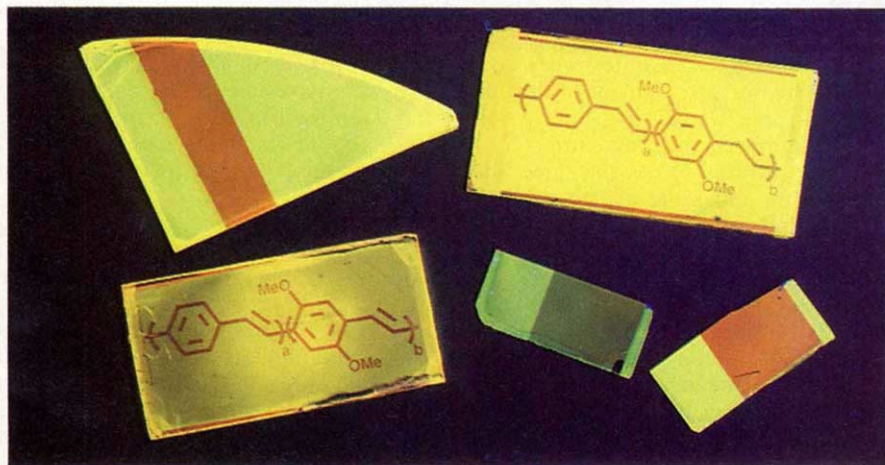


FIG. 2 Several samples of the new copolymer films prepared by Burn *et al.* fluorescing under ultraviolet light. The alternated block copolymer is made from two precursors, one of which has readily removed leaving groups to make the radiating, conjugated sequences. Differing treatments can leave the intervening sequences either nonconjugated or conjugated (but with a different band gap), so that one has a choice of radiating colour. In two samples, this choice has been used to etch the molecular structure of the fully conjugated copolymer into the film, so showing off another of the material's potentials.

with the smaller gap (GaAs). For a layer with a thickness of molecular size (1 nm or less) the electrons are confined to move within the layer. Perpendicular to the layer the electrons behave as a particle in a box and the energy levels no longer form continuous bands but are forced to have discrete values. These reduced-dimensionality structures have novel physical properties, exploited in new devices⁴. The use of quantum-well structures in lasers provides emission at shorter wavelengths than can be obtained from the bulk material. This is because emission occurs from the discrete energy levels which have an energy separation greater than the energy gap of the bulk material.

The behaviour of electrons confined in a small region accounts for the observed size dependence of absorption and emission wavelengths of both small conjugated molecules and semiconductor quantum wells. However, in other respects the structure and properties of the polymers described by Burn *et al.* and the III-V semiconductor quantum-well structures are very different. The latter are crystalline materials with regular, controlled variations in composition. The electrons are free to move in the plane of the layers and this two dimensional motion, which occurs with very high carrier mobility, is used to advantage in devices⁴. The polymers, how-

ever, are amorphous and the conjugated blocks vary in both size and separation and are randomly distributed in the polymer film. The behaviour of idealized polymeric quantum-well structures has been explored theoretically^{10,11}. However, the synthesis of polymers with precise block size and an ordered, chain-extended structure has not yet been

achieved. For the disordered materials studied by Burn *et al.*, the properties reported are closer to those of isolated small molecules than quantum-well structures. Despite this, these new materials are a further step along the road towards practical polymer-based electronic devices. The production of films capable of optical waveguiding and the definition of lateral structures by simple lithographic techniques (Fig. 2) illustrates the unique properties that can be realized with polymers, and demonstrates their potential for technological exploitation. □

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Unshellfish

CALCIUM carbonate seems to be one of the few waste products of biology. Once a shellfish, for example, has taken up dissolved calcium and turned it into a calcium carbonate shell, the resulting mineral is permanent. The white cliffs of Dover are not part of any life cycle.

Daedalus disagrees. He points out that in water containing carbon dioxide, calcium carbonate dissolves reversibly to give calcium bicarbonate. If the carbon dioxide is removed, the carbonate is re-deposited — stalactites, stalagmites and kettle scale are formed in this way. Molluscs, like shellfish, use an enzyme, carbonic anhydrase, to control and direct this reaction. But no enzyme can shift the position of a chemical equilibrium. With enough carbon dioxide in the water, the equilibrium would favour soluble calcium bicarbonate, and shellfish would be frustrated.

So Daedalus proposes that ships whose wetted surfaces get fouled with barnacles, and power stations whose cooling-water outlets become blocked with mussels, should blow their exhaust gases through the water. The carbon dioxide of combustion would dissolve. This would virtuously avoid releasing a greenhouse gas into the atmosphere, and (in the case of a ship blowing its smoke into the water ahead of it) would reduce drag by filling the water with small bubbles. Young barnacles and mussels would be unable to grow shells in the carbon dioxide-rich water; shipworms and other marine borers would find themselves impotent and toothless; all would drift away disconsolately to more congenial waters.

Animal lovers will be glad to know that the treatment should be quite humane. The water will still contain plenty of oxygen, so no sea creatures will be suffocated. They may even be stimulated by a bit of extra carbon dioxide, just as we are — oxygen with added carbon dioxide is used to revive victims of suffocation. Fish will not be crippled with arthritis by the dissolution of their skeleton, for its mineral component is calcium phosphate. They may, however, be disoriented. Fish otoliths (the little granules in the ear that give them their sense of gravity and inertia) are indeed made of calcium carbonate. If these dissolve in the carbon dioxide-rich water, severe vertigo and sickness may result.

DREADCO biologists are now testing these notions. They are growing clams, oysters and so on, in oxygenated water enriched with carbon dioxide, hoping to rear them intensively in shell-less and (sadly) pearl-less varieties for human consumption. The same treatment should turn snails into slugs, and ought to make fish seasick.

DAVID JONES

Fossilized eggs from Illinois

SIR — Apart from the egg capsules of chondrichthyans¹, fossilized eggs from the Palaeozoic are exceedingly rare. I report here the discovery of fossilized eggs in a siderite concretion from the Middle Pennsylvanian, Francis Creek Shale (Carbondale Formation, Desmoinesian Series, Westphalian C-D, about 300 million years old) in the Mazon Creek area of northeastern Illinois. Differential staining of the siderite matrix indicates that the circular ova were held together in a gelatinous string.

The area around Mazon Creek is one of the most important sources of late Palaeozoic fossils. The fossiliferous concretions are famous by virtue of the superb preservation of soft-bodied organisms, many of which are either unique to this area or represent the earliest or only known record of an important taxonomic group². The taxonomically diverse assemblage consists of more than 500 plant and animal species derived from two main faunal associations: the Braidwood biota, of brackish freshwater and coal swamp habitats; and the Essex biota, of near-shore marine habitats³. The fossilized egg strand (Royal Ontario Museum 47490) was collected by Karlene and Steve Ramsdell from a pit 11 spoil heap

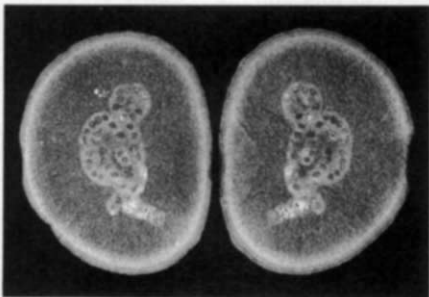
on the diameter of the ova are unknown. A gelatinous string, now seen as a light-coloured staining of the siderite, held the eggs in a single row. The preserved diameter of the once jelly-like sheath ranges from about 3 to 3.8 mm. Two other specimens are known (ROM 48443 and ROM 48444), but their gelatinous sheaths are not as clearly preserved as in ROM 47490.

Among extant organisms, several groups spawn egg strands. Some opisthobranch molluscs extrude eggs in gelatinous ribbons or sheets⁵. However, opisthobranch eggs range in diameter from about 40–400 µm, with most approximately 100 µm in diameter. Among vertebrates, at least five species within the Teleostei extrude egg strings or ribbons⁶. The largest number of species spawning egg strings that resemble those from Mazon Creek are to be found within the Amphibia⁷. Unfortunately, there are no known diagnostic features by which these fossilized eggs can be assigned taxonomically.

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Fossilized eggs from the Middle Pennsylvanian, Francis Creek Shale near Braidwood, Illinois (ROM 47490). Part and counterpart. Nodule maximum length is 58 mm.

of the now abandoned shallow shaft mines of the Peabody Coal Company. Most of the organisms from pit 11 are of the Essex (marine) biota, but in this area, a 2–5-km-wide zone of mixing between the Braidwood and Essex assemblages has been well documented⁴. Unfortunately, as a result of this faunal mixing, it is not possible to ascertain whether the eggs were derived from a marine or a freshwater organism.

The spawn, preserved in part and counterpart (see figure), split through the bedding plane on which it came to rest. Dark circular spots represent the remnants of about 40 eggs held in at least one but perhaps two strands. The diameter of the ova range from about 1.5–1.7 mm. The effects of preservation

No improvement in running?

SIR — B. J. Whipp and S. A. Ward (*Nature* **355**, 25; 1992) find that if they linearly extrapolate men's and women's running speeds, they obtain the result that women will overtake men in the marathon by the year 2000 and will overtake men in the 200-metre dash by the year 2050. The authors are apparently willing to extrapolate speeds in the 200-metre dash to about the year 2050. Using their graphs as published in *Nature*, by the year 2050, women will be able to run the marathon at a greater average speed than either men or women will be able to run 800 metres! This is certainly an astonishing result, and well illustrates the pitfalls of extrapolation.

My own view is that the reason the rate of improvement is greater for women than for men is that women's running is a younger sport. As the num-

ber of women runners increases from a small sample of the population to a more representative sample, it is natural that speed will improve. This improvement will come on top of the greater speeds arising from increasing body size and better training methods, which presumably are common to men and women, although not necessarily to the same degree. Marathon speeds have improved the most because it was only relatively recently that the marathon was considered to be a proper distance for women.

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SIR — The picture painted by B. J. Whipp and S. A. Ward (*Nature* **355**, 25; 1992), that women's running events are improving by leaps and bounds, is in sharp contrast with reality. Consider, for example, the prediction of a lowering of about 20 minutes for the marathon record within 6 years: the truth is that in the past 7 years the record has not improved by so much as a second.

Women's athletics has been declining recently: in the 1,500 metres, for example, the performances at 50th and 100th place in the 1984 world lists would, 5 years later, have given rankings as 16th and 46th, respectively. The current world record has stood since 1980. In the past 3 years, nobody has come within 6 seconds of it. Thus, contrary to Whipp and Ward's claim, the gap between women's and men's performance is widening (men's performance is fairly stable).

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SIR — If the predictions of B. J. Whipp and S. A. Ward (*Nature* **355**, 25; 1992) are to be believed, athletics administrators will soon be faced with a different set of problems over sex testing than those currently taxing them. In the Olympic Games due to take place in the year 2000, it may be potentially advantageous in terms of medal-winning for a woman marathon runner to masquerade as a man and race in the men's event. This advantage will progressively apply through the shorter events during the twenty-first century. In my view, the only equitable solution (which completely obviates the need for sex testing) is for the distinction between the sexes to be abandoned after 2050 in running events of 200 metres and beyond.

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HLA antigens and cervical carcinoma

SIR — Wank and Thomssen¹ claimed the association of HLA-DQw3 and HLA-DR5, the latter possibly as a result of linkage disequilibrium, with increased risk of squamous carcinoma of the cervix. They also noted a significant decrease in the frequency of HLA-DRw6. We have tissue-typed 65 patients (mean age 52.7 years, range 23–87) with histologically proven squamous cell carcinoma of the cervix (21, 27, 15 and 2 patients presenting with stage I, II, III and IV disease, respectively) and compared the HLA antigen frequencies with a local control group comprising 857 organ donors from the northwest of England; 347 females and 510 males with similar HLA antigen frequencies when divided by sex. The cervical cancer patients were referred for radiotherapy at the Christie Hospital from the same geographical area as the control group.

Our data, tabulated for HLA-DR and HLA-DQ, show no statistically significant associations of any HLA-A, -B, -Cw, DR or -DQw serologically defined antigen with the disease. In particular there is no increased frequency of HLA-DQw3 or decreased frequency of HLA-DRw6 in our patients, although the frequency of these antigens in control populations in both studies was similar. The frequency of DR5 in the United Kingdom population is about half that found in Germany but again there is no difference between the incidence of this

antigen in our patient and control groups. Furthermore, neither DR4 nor DR5, both of which are established to be in close linkage with DQw3, are increased in frequency in our patients. Comparisons for DR and DQ of probable homozygotes also show no difference in patient and control groups.

On the basis of our results, it may be premature to conclude that certain HLA haplotypes carry increased risk for development of squamous carcinoma of the cervix. Possible explanations of the differences between these similar sized studies may lie in an unintentional selection of either the patient or the control groups. Alternatively, different viral factors may be involved in the development of cervical cancers in the United Kingdom compared to southern Germany. It is also possible that there is microheterogeneity of DQ molecules in the two populations which might influence susceptibility. Molecular typing rather than serology may ultimately resolve whether DQw3 is truly a susceptibility gene for cervical cancer.

It remains a possibility that certain HLA antigens influence the development of squamous cell carcinoma of the cervix. Many immune responses are controlled by the genes of the major histocompatibility complex (MHC) and loss of MHC class I expression has been demonstrated in a significant proportion of cervical squamous carcinomas².

Further, our recent studies (S.S.G. *et al.*, manuscript submitted) demonstrate that most of these tumours, in contrast to normal cervical squamous epithelium, express HLA class II antigens. There may thus be several MHC factors contributing to the overall immune response to these tumours and to the viral agents that have been implicated in their aetiology.

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SIR — Our recent report¹ of a significant association between squamous cell carcinoma of the cervix and the HLA antigen DQw3, as defined serologically, suggests that genes of the MHC may influence the effectiveness of an immune response against this tumour, which is thought to be virally induced^{2,3}. Now, using sequence-specific oligonucleotides to define the specific DQ alleles⁴ associated with this susceptibility, we not only confirm our previous study but also find a preferential increase in the frequencies of the DQB1*0301 (40 of 50 patients) and *0303 alleles (9 of 50) in 50 DQw3-positive patients. Furthermore, in seven patients lacking a DQ3-related allele, five share a DQB1*0602 allele. Even more striking is the virtual absence of the DQB1*0302 allele in the DQw3-positive patient group (1 of 50) although it accounted for 36% of the DQw3 alleles in controls ($P < 0.0002$) (manuscript in preparation).

In the absence of monoclonal antibodies and alloantisera specific for HLA-DQw8 or HLA-DQw9, the serological distinction of these antigens is based on a series of reactions whose interpretation is often supported by information on the DR specificities, owing to the strong linkage disequilibrium of the genes encoding DR and DQ (ref. 5).

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HLA CLASS II PHENOTYPE FREQUENCIES

HLA-	IWC ⁴ (UK)	LC	Patients	χ^2	Significance
DR	N = 166	N = 857	N = 64		
DR1	26.9	19.3	15.6	0.85	NS
DR2	31.6	28.4	35.9		
DR3	24.8	28.8	23.4		
DR4	35.8	35.2	40.6		
DR5	10.5	12.7	12.5	0.021	NS
DRw6	14.9	23.6	15.6	1.69	NS
DR7	24.8	26.3	31.3		
DRw8	7.0	3.5	0		
DR9	1.2	1.9	1.6		
DRw10	0	1.4	1.6		
Single antigen	8.4	18.9	21.9		
DQ	N = 174	N = 65	N = 65		
DQw1	56.4	69.2	59.4		
DQw2	41.0	32.3	42.2		
DQw3	43.1	66.2	53.1	1.57	NS
Single antigen	33.1	32.3	45.3	3.36	NS

IWC(UK), International Workshop caucasian UK controls; LC, local caucasian controls; patients, people with squamous cell carcinoma of the cervix. Serological typing for 15 HLA-A, 22 HLA-B, 7 HLA-Cw, 11 HLA-DR and 4 HLA-DDQ antigens was performed by routine methods³ using a local panel of typing antisera obtained from our own screening programme, supplemented by sera obtained by mutual exchange with UK and international laboratories. All sera were thoroughly characterized by their reactions with local cell panels which had been typed using IHW antisera. Statistical analysis was by Fischer's exact test. No significant difference was found between patients and control groups. χ^2 values are given only for those HLA antigens that have previously been reported¹ as significantly altered in the patient group, or those which we found to have differences. The frequencies of DR antigens for the 65 DQ-typed local controls showed no significant differences from those of the patient group.

In our earlier study¹ we attempted to make a subtype assignment in only half of our DQw3-positive patients, and of the six patients that were assigned DQw8 (corresponding to *0302) only three were available for molecular typing: two have an *0303 allele which encodes the epitope associated with the DQw9 specificity, while the third represents the only patient among 50 with an *0302 allele.

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SIR — Wank and Thomsen¹ have suggested that there is an increased risk of developing squamous cell carcinoma of the cervix for individuals carrying the HLA-DQw3 antigen. They reported that of 66 patients tested, 87.7% carried HLA-DQw3 compared to 50.4% in controls (relative risk (RR) = 7.1, $P=0.0009$). Epidemiological studies have implicated a sexually transmitted agent in the aetiology of cervical cancer, and laboratory studies have established a strong association between certain human papillomaviruses (HPV 16, 18 and 33) and cervical cancer². Because viral antigens are presented to T cells by HLA molecules^{3,4}, an association with a specific HLA molecule would be of importance for our understanding of the carcinogenesis of the uterine cervix.

To see if the association reported by Wank and Thomsen could also be found in another and larger population of patients, we performed genomic typing of 213 patients with cervical cancer and 181 Norwegian caucasian controls⁵. The distribution of the different carcinoma types was as shown in the table. DNA samples extracted from blood were amplified by the polymerase chain reaction (PCR) using primers as described previously⁶, and hybridized with the following oligonucleotide probes detecting the DQB1 alleles encoding DQw3 variants: 5'-GG-CCG-CCT-GAT-GCC-GAG-3' (amino acids 54-59, hybridizing to alleles 0301 and 0303), 5'-GG-CCG-CCT-AGC-GCC-GAG-3' (amino acids 54-59, hybridizing to allele

DQB1 TYPING IN CERVICAL CANCER PATIENTS AND CONTROLS

Cervical cancer patients	No.	No. with DQw3
Squamous cell carcinomas	168	113 (67.3%)
Adenosquamous carcinomas	12	5 (41.7%)
Adenocarcinomas	25	13 (52.0%)
Others	8	4 (50.0%)
Controls	118	92 (50.8%)

0302), 5'-CGC-GTG-CGT-TAT-GTG-ACC-AGA-TAC-3' (amino acids 23-30, hybridizing to alleles 0301 and 0601).

We found that 67% of the patients with squamous cell carcinoma carried a DQB1 gene encoding HLA-DQw3, compared with 51% in the controls (RR=2.0, $P<0.002$; see table). The frequency in the control population was similar to that reported by Wank and Thomsen. Our findings thus confirm those of Wank and Thomsen, although the increase of DQw3 among patients with squamous cell carcinoma was less than that observed by them. The frequency of DQw3 among patients with cervical cancers other than squamous cell carcinomas did not differ significantly from that observed in our controls. We are now looking to see if this association is different among patients infected with different subtypes of human papillomavirus as well as the influence of the different DQw3-encoding alleles.

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Alzheimer's retraction

SIR — We would like to retract the paper entitled "Amyloid plaques, neurofibrillary tangles and neuronal loss in brains of transgenic mice overexpressing a C-terminal fragment of human amyloid precursor protein", which appeared in the 12 December issue of *Nature* (354, 476-478; 1991).

We (S. K. and J. W. G.) have successfully repeated the Southern and northern blotting results, thereby confirming the transgenic status of the mice and the

overexpression of the carboxy-terminal fragment of the human amyloid protein precursor gene. But we have been unable to reproduce the histopathological findings (originally performed by G.A.H.) depicted in Figs 3 and 4, showing amyloid plaques and neurofibrillary tangles detected by silver staining, immunocytochemistry and thioflavin S staining, in any of 12 transgenic animals recently studied.

Although we are continuing to evaluate our results, we believe it is prudent for the time being to retract these histopathological findings. The conclusion of the paper, that our transgenic animals constitute a useful rodent model of Alzheimer's disease, remains to be assessed by further study.

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Catalysis steps

SIR — Kallen *et al.* have reported the X-ray structure of the complex of human cyclophilin and the tetrapeptide *N*-acetyl-Ala-Ala-Pro-Ala-amidomethylcoumarin¹. Based on this structure, they propose a mechanism for the prolyl *cis-trans* isomerase activity of this protein that involves "Histidine 126 acting as a proton acceptor for a water molecule involved in nucleophilic attack on the carbonyl carbon of the Ala-Pro amide bond." Although this is an interesting hypothesis, it is at odds with our results on enzymatic prolyl *cis-trans* isomerization²⁻⁴.

First, a secondary deuterium isotope effect on k_c/K_m of 1.12 ± 0.02 is observed for the cyclophilin-catalysed *cis-to-trans* isomerization of *N*-succinyl-Ala-Gly(L,L)-*cis*-Pro-Phe-pNA ($L = H, D$)^{2,3}. The isotope effect is simply the ratio of rate constants, $(k_c/K_m)^H/(k_c/K_m)^D$, and reflects changes in hybridization or bond order at the carbonyl carbon of the Gly-Pro bond. A large, normal isotope effect such as this indicates that the carbonyl carbon of the Gly-Pro bond does not undergo nucleophilic attack during catalysis. Second, k_c/K_m is independent of pH for the cyclophilin-

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catalysed *cis-to-trans* isomerization of *N*-succinyl-Ala-Ala-*cis*-Pro-Phe-pNA (ref. 2). This observation strongly suggests that the imidazole of an active site His is not involved in catalysis. Third, there is no solvent deuterium isotope effect on k_c/K_m for the cyclophilin-catalysed *cis-to-trans* isomerization of *N*-succinyl-Ala-Ala-*cis*-Pro-Phe-pNA (ref. 2). This eliminates mechanisms involving general-acid/general-base catalysis such as that advanced by Kallen *et al.* in which His 126 is proposed to act as a general-base catalysis in isomerization.

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KALLEN *ET AL.* REPLY — In our paper on the structure of cyclophilin, a preliminary fit of the tetrapeptide substrate into the 2.8-Å electron-density map was modelled with the peptide in an all-*trans* conformation, with Arg 55 directly hydrogen-bonded to, and His 126 some 5 Å away from, the prolyl amide bond. This geometry suggested a mechanism in which a putative water molecule hydrogen-bonded to the histidine could mediate the *cis-trans* isomerase mechanism.

The experimental results summarized by Stein suggest that water and His 126 are not involved in the mechanism for *cis-trans* isomerization. Recent refine-

ment of the crystal structure using additionally collected high-resolution data ($R=17.8\%$ for all data to 2.3 Å) shows a good fit for the alanyl-prolyl amide of the tetrapeptide in the *cis*-conformation, with His 126 in close contact to the alanyl-prolyl carbonyl oxygen.

This higher-resolution X-ray refinement of the cyclophilin-tetrapeptide structure thus indeed does not confirm the presence of a bridging water molecule in the active site, but does show that His 126 (and Arg 55) are involved in substrate binding. Our conclusion that Arg 55 and His 126 are important residues in the *cis-trans* isomerase mechanism has also been further substantiated by site-directed mutagenesis experiments (M. Luyten *et al.*, in preparation). A publication presenting the fully refined structure and the detailed interaction of the tetrapeptide with cyclophilin is in preparation.

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Short-range flaws

SIR — Maddox¹ reports the claim by Suzuki² to have dealt with short-range divergence problems in quantum electrodynamics (QED) in such a way that the subtraction of infinite quantities from each other would be avoided. This result would render the so-called renormalization procedure obsolete; unfortunately a study of the derivation reveals some basic flaws in the argument.

Suzuki² introduces a unitary transformation which splits the hamiltonian H for the electron-photon system into two parts: H_1 and H_2 . Because both derive from the same photon field, these two operators do not commute. However, Suzuki explains his neglect of the corresponding commutator by (wrongly) invoking relativistic invariance. Such illicit manipulation is further demonstrated when the following surprising formula is proposed for the scattering S -matrix:

$$\exp \left[-i \int_{-\infty}^{+\infty} (H_1 + H_2) dt \right] \rightarrow \frac{1}{2} \left[\exp \left(-i \int_{-\infty}^{+\infty} H_1 dt \right) + \exp \left(-i \int_{-\infty}^{+\infty} H_2 dt \right) \right]$$

Even if the commutator were to vanish, one would get the S -matrix in factorized form and not as a sum. Anyway, one cannot neglect the commutator of H_1 and H_2 which allows quantum-exchange processes that are missing in Suzuki's work, giving rise to charge and mass renormalization.

What are 'renormalizable' theories? It may be that a finite theory could be derived to unify all the known forces. Such a possibility is offered by the so-called string theories that have undergone intense study³. In a truly finite theory with only one parameter with a physical dimension, it should be possible, in principle, to compute the mass of the electron or the coupling constant of QED, $\alpha = e^2/\hbar c \sim \frac{1}{137}$. In contrast, in a renormalizable theory such as QED, there are divergences that are absorbed in parameters such as electron mass and coupling constant α , adjusted to their experimental values. In this scheme, QED would be an effective theory for the low-energy limit of a more fundamental theory.

In condensed-matter physics, the application of field theory to the study of critical phenomena has also emphasized the nature of a renormalizable theory⁴. It emerges as the long-wavelength limit

of a microscopic theory containing a small physical length scale that suppresses the short-range divergences. In a solid-state system, the lattice spacing sets an upper bound to the allowed momenta in otherwise divergent (Feynman) integrals. In polymer physics the size of the chemical bonds provides this scale, much smaller than the size of the macromolecule⁵.

Another striking example can be found in quantum chromodynamics (QCD). This gauge field theory, which is renormalizable, provides a description of strong interactions, the experimental successes of which have been reported⁶. On the theoretical side, this field theory has been recently derived⁷ as the low-energy limit of a particular finite string theory. The infinities of the limiting renormalizable field theory, that is, QCD, appear in the formal mathematical limit where the length scale built into the string theory tends to zero. In unified theories based on strings³, this length scale is expected to be the Planck length constructed from Newton's constant G , $\hbar$ and c , and which is of the order of 10^{-33} cm.

Returning to QED, it should be the effective description of electromagnetic processes at distances much larger than the Planck length. Presently, its closed form is relativistically covariant, and, after renormalization, leads to finite answers to all orders in perturbation theory. The perturbative calculation of the gyromagnetic ratio of the electron (the Landé factor), in excellent agreement with experiments, certainly include mass and charge renormalizations. Such effects are absent in Suzuki's paper as his neglect of the cross-processes leads to no mass renormalization at all. It is implausible that a simple trick could fundamentally alter the present status of QED, the most accurate physical theory ever built.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references.

An unreliable history

Simon Mitton

Stephen Hawking: A Life in Science. By Michael White and John Gribbin. Viking: 1992. Pp. 304. £16.99. To be published in June in the United States by New American Library at \$23.

STEPHEN Hawking has been poorly served by this biography, which mixes good popular science with flawed history. Hawking's achievements in physics are clearly remarkable, his battle against a crippling disease is inspirational, and even now the success of *A Brief History of Time* is inexplicable. This is an intensely interesting story; sadly, the target is missed.

Hawking's research has principally dealt with the very early Universe, the physics of black holes and the search for a unified theory of everything. His intriguing discoveries include such counter-intuitive concepts as the notion that 'mini' black holes can evaporate. In 1974, this breakthrough, now known as the Hawking effect, showed that quantum physics and general relativity could give new information on the workings of the Universe. As White and Gribbin explain, the work on exploding black holes then led Hawking to concentrate on the singularity that led to the birth of our Universe. By the mid-1970s, he was firmly established as one of the leading physicists of his generation.

Since then, Hawking and his school have searched for a unified theory in which all of physics would be described by a single set of equations. The 'end of physics' is sought in the first glimmer of our Universe, a scrunched-up opportunity space with numerous hidden dimensions. This is serious physics, which could have a profound influence on gravitational physics and cosmology.

Many of those who bought *A Brief History of Time* have not read it because it is difficult. White and Gribbin succeed in giving a clearer description of the physical Universe that interests Hawking. The chapters devoted to modern physics are done well and they capture the excitement of scientific investigation of the most fundamental kind. With the biography entirely stripped out, the remaining material would make a book of the sort that James Jeans was writing in the 1930s or Paul Davies today: a good story to get through in one evening, with plenty to talk about the next day.

The problems arise with the biographical element and the supporting structure. It is all too clear that the authors were working with inadequate and poorly researched material. The quotations attributed to Hawking, his family and most of his collaborators are largely taken from existing sources, such



Hawking — Making hard physics a required purchase.

as the television programme *Master of the Universe* and interviews given to launch *A Brief History of Time*. New material is based on a few interviews with people (including myself) mainly outside the penumbra. Some of this is little more than tittle-tattle, such as small incidents on train journeys from London to Cambridge, Hawking's undergraduate pranks, subjective statements about Hawking, and his tangle with a college bursar over the charge for a room.

More seriously, the book contains many errors. We are told that Maxwell wrote his equations "in order to explain radio waves which had recently been discovered". In fact, Maxwell's equations were published in 1864, and in 1879 Helmholtz suggested to Hertz the experiments that led to the detection of radio waves in 1888. In the biography of

a man who, like Maxwell, is searching for unification of the forces, this error is unfortunate. And what are we to make of the statements that the Hawking family are lifelong members of the Labour party, when a page is devoted to describing his painting "VOTE LIBERAL" graffiti?

The authors resort to the literary device of fiction based on fact because of the absence of new evidence about Hawking. In a distortion of the Oxford of the 1960s, clichés abound: dukes' daughters in ball gowns, lazy days punting, "hung-over young men and occasional women" at breakfast. Scholarships were not offered by the university, and the financial element of college awards was nominal. Instead of searching out

the examination Hawking sat, the authors invent a "typical question".

Cambridge gets the same treatment. It is very unlikely that only the final chapter of Hawking's thesis saved him from failure to be awarded a PhD. It is false to say that the role of a college fellow "has changed little since Sir Isaac Newton's time" and that the duties of fellows are minimal apart from research. I do not believe that Hawking regards physics as a "perfect displacement activity", and he is not head of his department.

The time axis of this book is punctuated by news flashes that demand knowledge of British history: we are told that as Hawking thinks, in 1982, peace protesters are on Greenham Common, just in case we cannot locate the event in spacetime. Some of this too is sheer invention. This book describes a walk I never made, during which a car I never saw pulled up, and I never ate tea and biscuits for lunch.

Hawking wrote a book that changed popular science publishing. As a result, booksellers are much more confident about displaying science books. To be on the bestseller list for three years is a stupendous achievement. A chance is missed here to explain why the book has sold so well. If I had acquired the rights I would have been pleased with sales at one per cent of those achieved so far. What is it that pushes a product into the 'must have' category? How can hard physics be a required purchase? For answers to these and other questions we must await a more reliable biography. □

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Spark gaps

Willem D. Hackmann

The Ambiguous Frog: The Galvani–Volta Controversy on Animal Electricity. By Marcello Pera, translated by J. Mandelbaum. Princeton University Press: 1991. Pp. 203. \$29.95, £18.

IN 1745, fellows of the Royal Society of London were astonished to learn about a novel experiment performed at Leipzig in which sparks were drawn from the lips of an electrified woman by anyone who tried to kiss her. They immediately set about acquiring the electrostatic generator used to produce this 'electric kiss', in no doubt that when the apparatus

that the muscle fibre and the enclosed nerve acted in the manner of a Leyden jar (a primitive capacitor). The storm Galvani provoked among physicists, physiologists and physicians can be compared with the political storm that arose at that time over Europe. Volta disagreed profoundly with Galvani's ideas, suggesting instead that electricity was simply an agent of nervous stimulation.

The Ambiguous Frog is not, as Bernard Cohen makes clear in the foreword, a conventional recasting of a familiar story. Instead, Professor Pera introduces the Galvani–Volta controversy as a fable of how a dissected frog when requested to reveal its inherent electricity made a mockery of a doctor and a physicist, and revealed their hidden metaphysics instead. What Pera brings out of this

scientific fable very clearly and cleverly is that we have here a conflict not just of two different research programmes but of two different gestalten in the same area of observations: the electrophysiological gestalt of Galvani, the anatomist, versus the electrophysical gestalt of Volta, the physicist.

Both scientists performed crucial experiments that validated their own theoretical framework. Galvani

proposed a naturally unbalanced animal electricity, Volta an artificially unbalanced electricity in animals. This induced Volta to develop his special contact theory of electricity of metals into a general theory of contact electricity in which all conductors, metallic or not, are electromotive. His general theory of contact electricity had two important consequences: first, it stated the same thing as Galvani's animal-electricity theory but with a different interpretation about the realm to which the phenomena belonged; second, it resulted in 1800 in his momentous discovery of the voltaic pile, the first electrochemical battery, the harbinger of profound conceptual and technological advances in physics. Because of the theoretical positions they had taken up, Galvani never explored the possibility of the existence of the 'current of injury' in these phenomena, and Volta failed to see the importance of the chemical effect in the action of his pile.

The key point of Pera's scientific fable is that scientific controversies are not usually won on facts alone, despite what Volta himself wrote on the subject. The protagonists each have their own 'interpretative theories' that specify the phenomena as they see them. In this case, because neither Galvani nor Volta accepted his adversary's gestalt, there could be no genuine crucial experiment

to decide between the competing theories. Pera likens this situation to the interpretation of ambiguous shapes, such as Joseph Jastrow's duck–rabbit, in which the same perceptive material is self-organized into different forms, the one that is seen depending on the gestalt adopted by the observer. In the case of the frog leg, do we see an organic capacitor or a physical electromotor? As Pera points out, although in these scientific episodes we go beyond the language of facts and inductive logic, we can still conduct a rational discussion. □

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What ghost in the machine?

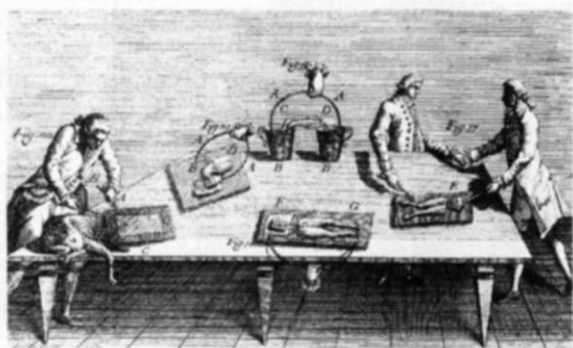
Tim Shallice

Consciousness Explained. By Daniel C. Dennett. Little, Brown: 1991. Pp. 511. \$27.95. To be published in the United Kingdom by Allen Lane The Penguin Press on 16 March at £20.

TWENTY years ago, scientists rarely referred publicly to consciousness. Today the taboo has been lifted. Cognitive scientists and neuropsychologists now describe many phenomena involving awareness and speculate widely about consciousness, although this newly found confidence has not been matched by much gain in theoretical understanding.

Philosophers of mind have responded in two different ways. Some have built Chinese walls to keep their territory inviolate from the crudely functionalist cognitive scientists. But others, above all Dan Dennett, have responded positively and attempted to adapt their theorizing to developments in cognitive science and neuroscience. Over the past 20 years, Dennett has written three important works on consciousness — *Content and Consciousness* (1969), *Brainstorms* (1978) and now, most grandly of all, *Consciousness Explained*. The first book, apart from taking the functionalist approach, shows little direct scientific influence. *Consciousness Explained*, by contrast, is full of fascinating accounts of scientific findings. The philosophical ideas it contains are influenced by a range of disciplines from evolutionary theory through neurophysiology and cognitive psychology to artificial intelligence. This, then, is a book to which scientists interested in the philosophy of mind will naturally warm.

"What we have to understand", Den-



Animal electricity — from Galvani's *Commentarius* of 1791.

arrived in London "our own Country-Women will be found to have as much Fire in their Lips as any of their Sex in Germany". During the next decade, the human body formed an integral part of the electrical machine, acting as a collector of charge when suspended by silk cords or insulated by a cake of wax. The physiological reactions to electric shock were obvious and there was much debate about the seat of electricity in the body. Early conjectures compared the brain to the electrostatic generator, and the nerves to the conductors or wires through which electricity and volition were transmitted. Others assumed that electricity in animals was produced by ingested food or the friction caused by the circulation of the blood. New impetus was given to these ideas by the discovery of the electric organs of the torpedo fish and, in 1781, by Galvani's chance discovery of the sudden twitching of dissected frog legs when an electrical machine was discharged nearby.

In a series of carefully executed experiments, Galvani found that he could produce the same spasmodic convulsions when the frog formed part of a circuit containing one or more pieces of metal, and in 1791 he not unnaturally postulated the existence of animal electricity as distinct from the common variety studied in the laboratory. He suggested

nett argues, "is how a Joycean . . . serial phenomenon can come to exist . . . in the parallel hubbub of the brain." He maintains that philosophical fallacies frequently arise from viewing phenomena at the serial level, and that these phenomena would be more appropriately viewed from the perspectives of the parallel one. Take his dissolving of the well-known paradoxes described by the neurophysiologist Benjamin Libet. Libet attached scalp electrodes to his subjects to measure their brain activity, and then asked them to note exactly when in time they made a spontaneous decision to flex, say, their wrist. In each case, Libet found that the start of an electrical response known as the *Bereitschafts* potential occurred 250–400 milliseconds before the subject was conscious of making the decision. Such experiments led Libet to postulate a mysterious, almost dualist, gap between the real physical events in the brain and their conscious correlates. Giving many original illustrations, Dennett argues that Libet is in the wrong conceptual domain; at this time-scale and grain of analysis there is no Great Divide between before and after coming into awareness. The analogy that Dennett draws is with the dissemination of an article in today's electronic-mail age; the article can be available in numerous versions between an early draft and its appearance in a journal. There is no instantaneous appearance fully formed in the public arena.

Dennett gives impressive treatment to philosophical puzzles such as John Searle's Chinese Room and the concept of self. But when he moves to the processing level, he denies that consciousness relates to anything special about the cognitive systems of humans or higher animals if these systems are considered as machines. He believes that many partially separable processing systems exist, which fits the standard neuropsychological position, but that none has any central role that would fit it for the seat of consciousness. His argument, however, floats above the level of the many particular theories that have been put forward. No account at all is taken of theories that relate consciousness to the operation of a set of processing systems.

Instead, Dennett describes human consciousness as "a huge complex of . . . meme-effects (in the Dawkins sense) . . . that can be best understood as the operation of a 'Von Neumannesque' virtual machine implemented in the parallel architecture of a brain that was not designed for any such activities" (my italics). Here "virtual machine" is used in the computer-science sense — an environment for programs running within a computer that makes it seem to each of them that it has an appropriate



FOLLOWING in giant footsteps — Thousands of dinosaur tracks have been found in recent years. Once thought of as rare and interesting curiosities, the tracks are now recognized as important scientific data that can tell us much about the day-to-day activity of many extinct species. Dinosaur tracker Roland T. Bird spent the 1930s and 1940s working as a field collector for the American Museum of Natural History. He is pictured here at the Paluxy River site, Texas, now Dinosaur State Park, where he discovered some of the first tracks of brontosaurus in 1938. The picture is reproduced from *Tracking Dinosaurs: A New Look at an Ancient World* by Martin Lockley, a nontechnical, well-illustrated guide covering dinosaur locomotion, behaviour, ecology and environmental impact. Published by Cambridge University Press, price £27.50, \$39.50 (hbk), £9.50, \$14.95 (pbk).

machine. An example would be a LISP virtual machine being implemented in the C programming language, with the machine it runs on being conceptually irrelevant. The hardware–software analogy is pushed to its extreme: "human consciousness is too recent an innovation to be hard-wired into the innate machinery . . . and its successful installation is determined by myriad microsettings in the plasticity of the brain . . . Besides the idea of the *user illusion* of a virtual machine is tantalisingly suggestive."

Unfortunately, we are given no real help in understanding how the virtual machine might come to run on this parallel hardware. Dennett discusses complex artificial-intelligence problem-solving programs that learn, such as the SOAR program of the Carnegie Mellon University group, and that have partly parallel architectures. But the serial-processing aspects of SOAR are a direct consequence of its design specification, not a product of its learning environment. More critically, he takes no account of the way in which several cognitive processes have a group of properties. They operate on or produce only a limited set of items at a time, but these can have many different types of content. They have a neurobiological basis. They seem to require awareness or to be intimately linked to it. They include attention, memory retrieval, error correction, intention formation and execution and the end-points or beginnings of language comprehension and production. Dennett would appear to maintain that the link between such processes and

'awareness' is merely a complex manifestation of our culture, but to a neuropsychologist this seems implausible.

He discusses in detail a few critical phenomena such as 'blindsight', where the rare patient with a lesion of the occipital lobe cannot consciously perceive stimuli in part of the visual field but can respond appropriately, for instance by pointing at the stimuli when 'guessing'. The visual information to which blindsight patients have access is held to be so "paltry" that they cannot base intentional actions on it. To deny being able to see is the only reasonable account they can give. Maybe, he speculates, they could be trained to use actively the information that they have. Then, maybe, they would say that they see. It seems though more likely to be the nature of the information and its structural base, in particular the use of extrastriate pathways in the brain, not the amount of information, that leads to their saying that they do not see. They can, after all, make an acuity judgement much better than a nearly blind person.

We are left then with an attractive tour through the science and philosophy of consciousness, a splendid dissolving of the supposed problems that functionalist accounts of consciousness are held to have. Yet to dissolve away consciousness itself as a biological phenomenon in the process is too easy a solution. □

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Putting us to ignorance again

Peter T. Landsberg

Thermodynamics: Foundations and Applications. By Elias P. Gyftopoulos and Gian Paolo Beretta. *Macmillan, Inc., New York: 1991. Pp. 658. \$66.*

LET us take the view that the *basic* ideas to be taught in thermodynamics should be the same for all students, be they engineers, chemists or physicists, while the applications should differ so as to be most appropriate to the students' needs. What, then, should constitute the basic framework? My own answer would be: (1) a phase space in which points represent equilibrium states and curves represent quasistatic (that is, reversible) changes; (2) the ideal gas and some other simple systems; and (3) a clear understanding of inexact and exact differentials.

The students at the Massachusetts Institute of Technology, who have attended at least part of the course presented in this book, are by no means all engineers. In fact, the authors do not seem to address themselves to any particular students. I would expect, however, that engineering students are the most suitable readers. But there is one caveat: they should not know the subject beforehand, because the ideal readers are, according to the authors, those "without much background in thermodynamics".

So reviewers are not really very suitable readers because they "know too much". The authors go on to explain that "experienced readers... may be appalled when they read that thermodynamics applies equally well to macroscopic and microscopic phenomena, that entropy is equally well defined for equilibrium and non-equilibrium states, and that temperature is... useful also for... a single particle... The new perspective requires... a subtle and demanding reconsideration of basic premises...". Thus there is little in the book on the first two points of my desiderata, and almost nothing on the third. But there are some challenging new points of view. The emphasis is away from mathematical concepts. This is not because the authors do not like to touch mathematical physics — I need only refer to their research papers on "quantum thermodynamics" published in *Nuovo Cimento* and elsewhere in the mid-1980s. (Their proposed union of mechanics and thermodynamics even attracted a leading article in *Nature* back in 1985 (316, 11).)

The unusual nature of the authors'

exposition is illustrated by the fact that the first and second laws of thermodynamics appear in chapters 1–4, whereas adiabatics and entropy are introduced only later (in chapters 5 and 7 respectively). It may be that beginners will take strongly to the book; and so they should, considering that the course on which it is based has been taught for 20 years. The authors have worked outstandingly hard: there are many tables and numerical data and plenty of problems. It seems churlish to hesitate to give the book a full-blooded recommendation.

But I do hesitate, in part because of details such as the definition of reversibility (page 59), the alleged achievement of perpetual motion in the laboratory (page 39) and the absence of a reason why the ideal gas cannot exist near $T = 0$ (page 325). More excusable for non-British authors is the description of Lord Kelvin as an English physicist, even though I fancied I saw him turn in his grave on being thus described. (He

was born in Belfast of Scottish parents and held university chairs in Scotland.)

The length of the book (it seems occasionally verbose) seems to be due to the authors' desire to cover both graduate and undergraduate courses, both introductory and advanced. However admirable the book's concept, its lack of references to research papers of even the 1960s or 1970s means that a graduate course would require yet more material to be added to an already long exposition.

In fact, the atmosphere of the book gives the impression of thermodynamics being a closed rather than a continuing subject. Perhaps the authors could present their philosophy of the subject forward more clearly by putting it into a shorter book next? □

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Killing fields

Gwyn T. Williams

Apoptosis: The Molecular Basis of Cell Death. Edited by L. David Tomei. *Cold Spring Harbor Laboratory Press: 1991. Pp. 336. \$44 (pbk).*

ALTRUISM is easy to justify for cells — the death of some for the benefit of the whole organism makes perfect genetic sense. Nevertheless, in accepting the idea that biological systems can be controlled by the modulation of cell death, scientists have had psychological as well as technical barriers to overcome. The current interest in active cell death would suggest that the importance of the process is now widely appreciated. The fundamental implications of the phenomenon account for the broad range of subjects represented in this book, which originated from a meeting held in 1990.

Several chapters deal with apoptosis as a whole, and the reviews by J. F. R. Kerr and B. V. Harmon and by S. R. Umansky serve as an especially useful introduction to the field. The rest of the book is concerned mainly with cell death in cancer development and treatment; there is an increasing awareness that cell populations can grow without increasing their proliferation rate provided that the rate of cell death is reduced. It has now been clearly shown that failure or suppression of physiological apoptosis can have an important role in cancer development. On the other hand, several contributors describe attempts to eliminate malignant cells through inducing apoptosis by irradiation, cytotoxic drugs, manipulating the hormonal signals received by the cell or engaging specific

cell-surface receptors with antibody. The true effectiveness of these procedures is not yet known.

Other authors cover areas of immunology. The reviews of T-cell-mediated killing by R. C. Duke and thymocyte death by D. J. McConkey and S. Orrenius address research areas that have been greatly influenced by the concept of cell death as a closely controlled, active process.

Neurobiologists have long appreciated that programmed death of developing neurons is suppressed by specific growth factors. A. C. Server and W. C. Mobley's discussion of neuronal death is very helpful for those outside the field. The authors emphasize that an understanding of active cell death in other lineages should prove to be valuable in establishing the significance of the process in neuronal development and degeneration, despite the idiosyncrasies of neuronal death.

The book provides a valuable overview of apoptosis, even though there are important areas that it does not fully cover. The molecular genetics of the precisely programmed cell death that occurs during the development of the nematode *Caenorhabditis elegans*, and the role of the proto-oncogene *bcl-2* in suppressing cell death in both physiological and pathological situations, for example, certainly deserve more extensive discussion. Such deficiencies are inevitable in such a rapidly developing field and may well be rectified in a second volume on the subject, now in preparation. □

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Molecular container compounds

Donald J. Cram

Rigid, hollow organic host compounds have been designed and synthesized with interior cavities large enough to incarcerate organic guest compounds. Some of these hosts are closed-surface spheres whose guest molecules are permanently incarcerated during synthesis. Other hosts contain portals in their shells, shaped to allow the passage only of complementary guest molecules between the inner and bulk phases. The inner phases uniquely constrain guest movements, provide a medium for reactions, and shelter molecules that self-destruct in bulk phases.

BIOLOGICAL chemists are like historians who discover, characterize and interpret evolutionary phenomena. Organic chemists resemble novelists whose characters and plots are inspired by the play of the imagination on actual people and events. The ideas and experimental results described here are products of synthetic and physical organic chemistry, inspired by the central part played by complexation of organic compounds in the molecular machinery for enzymic catalysis, molecular transport, genetic expression and immunological response.

Complexes are composed of two or more compounds held together in a describable geometry by hydrogen bonding, ion pairing, π - π binding dispersion interactions, van der Waals attractions, metal ion ligation or solvophobic phenomena. These forces involve binding energies much smaller than the 50–100 kcal mol⁻¹ that connect atoms in organic compounds. To accumulate a binding energy in solution of 2–20 kcal mol⁻¹, complexing partners require simultaneous binding at multiple sites that are stereoelectronically complementary to one another. Generally, acceptor sites of enzymes and nucleic acids possess convergent, concave surfaces, whereas their substrates or inhibitors possess divergent binding sites as parts of convex surfaces. We apply the term 'host' to synthetic compounds that are the counterpart of acceptor sites of biological chemistry, and the term 'guest' to compounds that are the counterpart of substrates or inhibitors for the acceptor sites¹.

Structural recognition in complexation depends on stereoelectronic complementarity of receptor (host) and substrate (guest), and can be measured by the free energy of association of complexing partners. The strength of binding is controlled by the kinds and numbers of simultaneous contacts between binding sites, the degree of complementarity of the binding partners, the extent to which the host and guest are preorganized for binding in their uncomplexed state, and solvation effects. Most host-guest complexation studies have been done in organic solvents, most of which are potential guests. Aggregates of solvent molecules resemble hosts. Complexation of hosts and guests is therefore highly dependent on solvent character, as solvation and complexation compete².

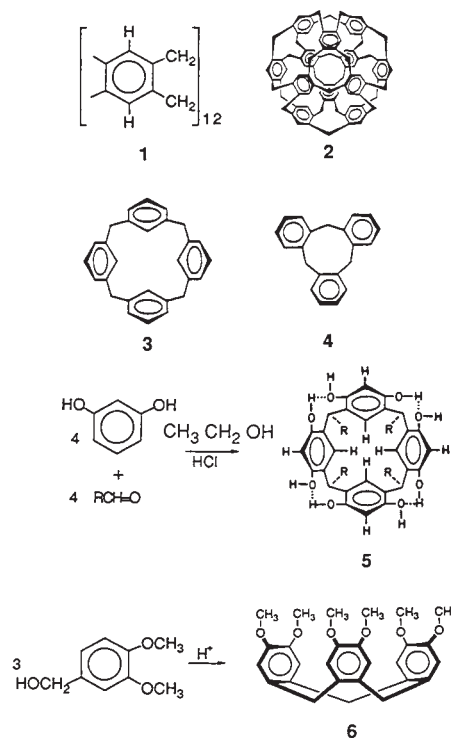
Here I review the newly discovered phenomenon of constrictive binding, in which guests are incarcerated in hosts and held there by mechanical means, as well as by dipole-dipole interactions and solvation. Extensive reviews of complexation involving other types of binding are available^{3–6}. The exciting feature of rigidly hollow hosts is that they enclose inner phases (interior cavities) which can limit guests' degrees of freedom, provide molecularly sized loci for guests' reactions and protect guests too reactive to be observed in bulk media.

Constrictive binding can be visualized with the help of Corey-Pauling-Koltun (CPK) plastic molecular models, whose design was based on the crystal structure determinations of many organic compounds⁷. Throughout the design and synthesis of complexes, CPK models served as a compass and crystal structure determinations as a sextant in an uncharted ocean of possible binding partners (more than 10 million characterized

organic compounds were recorded by *Chemical Abstracts* by 1991). Very few of these synthetic organic compounds contain enforced concave surfaces, although such compounds abound in biological chemistry.

Prototypes for hosts

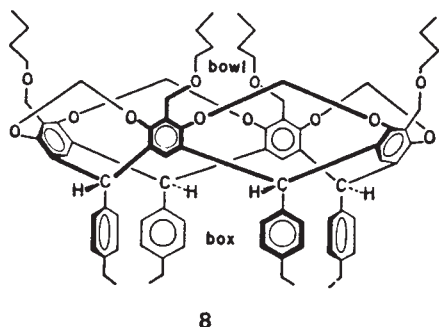
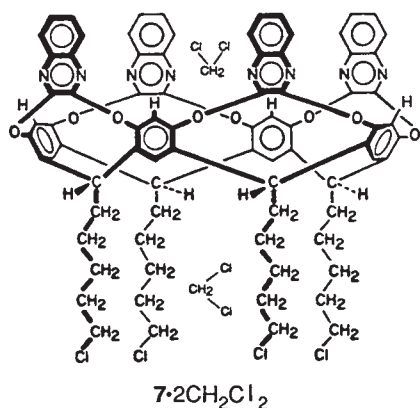
The inner faces of closed-shell, spherical compounds provide an extreme example of compounds containing concave surfaces. CPK models can be assembled to form spheres composed of 12 benzene rings and 24 connecting groups (for example, CH₂, O, S) with structures exemplified by molecular formula 1 and structural formula 2. The model is about the size of an American football, whose shell encloses a cavity about the diameter of a



grapefruit. Molecular models of many organic compounds easily fit inside 2. The skin of 2 merges six saucer-shaped units of structure 3 with eight saucer-shaped units of structure 4. Fortunately, compounds such as 5 (ref. 8) and 6 (ref. 9) had already been prepared. These concepts, coupled with the viable syntheses of structural variants of 5 and 6, facilitated my work.

Cavitands and cavitplexes

Cavitands are host compounds whose enforced concave surfaces are large enough to form complexes with organic guests without steric inhibition of either the complexation or the decomplexation process. Cavitplexes are their complexes. An example based

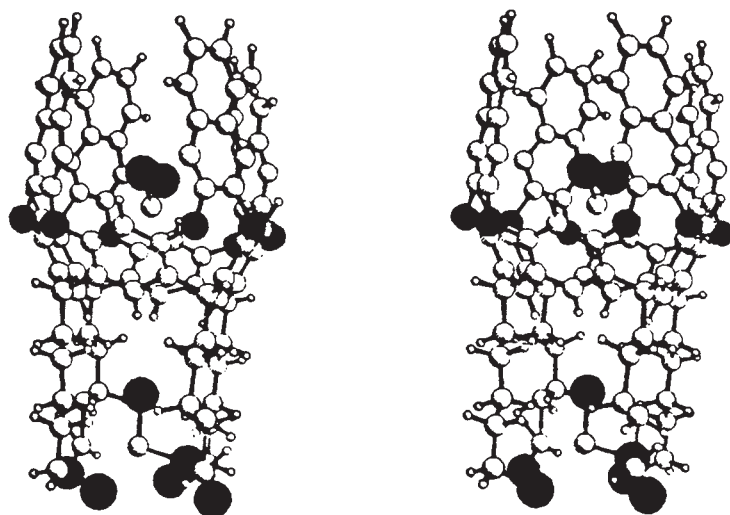


on the rigidification and extension of **5** is **7**, which was prepared in one step from **5** with $\text{R} = (\text{CH}_2)_5\text{Cl}$. Because of its large, open-top, vase-like structure, compound **7** complexes almost any solvent it contacts, complicating binding studies in solution. The four long, conformationally mobile feet appended to the vase render this cavitant and its caviplaxes soluble in organic solvents. Complexes of **7** in the solid state require temperatures in excess of 150°C and high vacuum to release their guests, whereas in solution, one guest can be easily washed out by a second¹⁰.

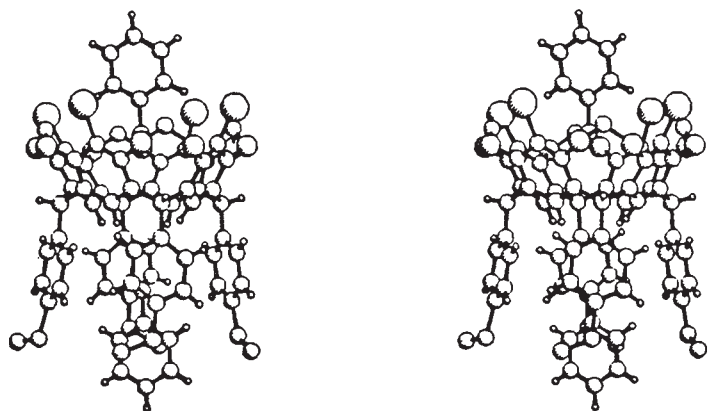
The crystal structure of $7 \cdot 2\text{CH}_2\text{Cl}_2$ shows that one molecule of CH_2Cl_2 acts as a guest in the vase part of the caviplax, while the second molecule acts as a solvate between the four feet that help the molecules pack into a lattice. Most crystal structures of these complexes are obtained as solvates, frequently with the solvent disordered¹⁰.

Cavitant **8** contains two different cavities, one bowl- and the other box-shaped. The four CH_3CH_2 'toes' on the rigidly organized phenyl feet, and the four $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$ 'braids of hair' appended to the bowl, extend the cavities and render **8** soluble in organic media. When crystallized from $\text{CH}_3\text{C}_6\text{H}_5$, **8'** (**8'** is **8** with four Br groups substituted for the four $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$ groups) gave $8' \cdot 2\text{CH}_3\text{C}_6\text{H}_5$, each of whose cavities contains a $\text{CH}_3\text{C}_6\text{H}_5$ molecule with its methyl group facing inwards¹¹.

Association constants (K_a) for **8** binding $\text{CD}_3\text{—C}\equiv\text{N}$ (CD_3 facing inward) were determined in CCl_4 (a solvent too large for the cavities) by proton magnetic resonance (^1H NMR). The K_a values of bowl and box were, respectively, 313 and 159 M^{-1} at 0°C . From the temperature dependence of K_a were obtained ΔH° values of 6.2 and 6.4 kcal mol^{-1} for the bowl and box, $-T\Delta S$ values (at 0°C) of -3.1 and $-3.7\text{ kcal mol}^{-1}$, and ΔG° values of -3.1 and $-2.7\text{ kcal mol}^{-1}$, respectively. The relatively high enthalpy of binding of more than -6 kcal mol^{-1} for each cavity is thus half cancelled by the entropy penalty of freezing out degrees of freedom of the guest on complexation, to provide a net free energy of binding of $\sim -3\text{ kcal mol}^{-1}$. Such binding



Stereoview of $7 \cdot 2\text{CH}_2\text{Cl}_2$, Os and Cls darkened

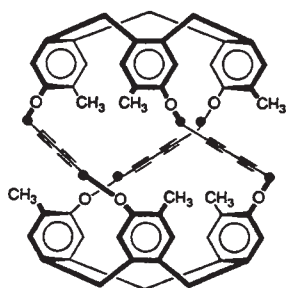


Side stereoview of $8' \cdot 2\text{C}_6\text{H}_5\text{CH}_3$

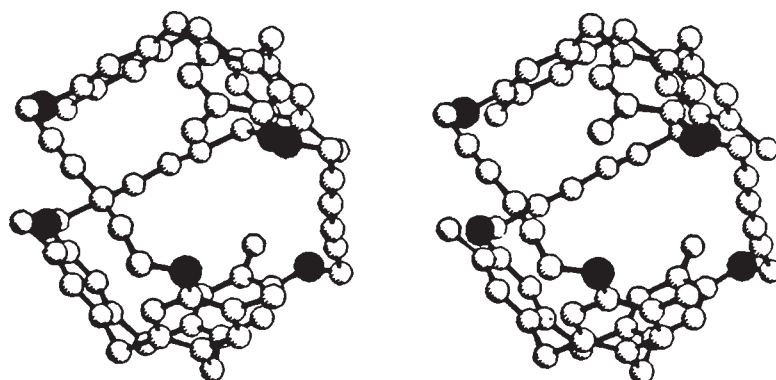
is rather large when we consider the small areas of contact between host and guest, and that only dipolar dispersion forces are involved. This binding provides greater than 50% occupancy of each cavity at low concentrations of host (0.0022 M) and guest (0.052 M)¹¹.

A third cavitant (racemic **9**) is based on the attachment of two rigid structural units of **4** to one another through three linear $\text{CH}_2\text{—C}\equiv\text{C—C}\equiv\text{C—CH}_2$ bridges. The identity of racemic **9** was established by its crystal structure, a stereoview of which is formulated without its hydrogens, and with its oxygens darkened. The interior of host **9** is occupied by disordered CH_2Cl_2 , which is omitted in the crystal structure representation¹².

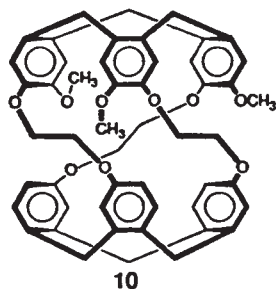
An interesting feature of the crystal structure of racemic **9** is its spherical shape, produced by rotation of its northern hemisphere around its polar (C_3) axis by 120° with respect to its southern hemisphere. The three linear $\text{CH}_2\text{—C}\equiv\text{C—C}\equiv\text{C—CH}_2$ bridging groups lie along the rims of the two polar caps, each composed of three benzylenes. A total of 88 atom-to-atom close distances (van der Waals distance plus $0.20\text{ \AA}$, or less) are created by this 120° twist, which also provides the conformation of least inner volume for this unusual host. The compound is chiral and has D_3 symmetry. As in its crystal structure, CPK models of **9** in its compact form contain no portals, whereas in its extended form, three large portals exist in which the axes of the three linear $\text{CH}_2\text{—C}\equiv\text{C—C}\equiv\text{C—CH}_2$ groups are nearly parallel to the polar axis of the molecule. Molecular models of



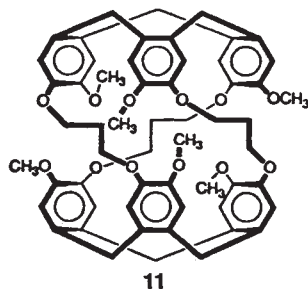
9



Stereoview of the crystal structure of 9



10



11

the extended form are large enough to allow models of molecules such as C_6H_6 or $CHCl_2CHCl_2$ to enter and depart with ease¹².

For binding studies of 9, $(CCl_3)_2CO$ was chosen as solvent, because the model of this solvent can enter the most extended form of 9 only with great difficulty. At $-20^\circ C$ in this solvent, racemic 9 binds the following guests with association constants K_a (M^{-1}) and ΔG° ($kcal\ mol^{-1}$), respectively, as follows: $CHCl_3$, 77,000, 5.7; $(CH_3)_3COH$, 16,000, 4.9; CH_2Cl_2 , 13,000, 4.8;

$H_2C=CH-CH_3$, 6,000, 4.4; cubane, 4,000, 4.2. With these guests at $-20^\circ C$, the exchange between complexed and free guest was slow on the 1H NMR timescale. In contrast, estimates of the K_a values for C_6H_6 and $CHCl_2CHCl_2$ were 1,000 and between 10 and $100\ M^{-1}$, respectively (1H NMR titrations at $21^\circ C$)¹².

Examination of CPK models of the caviplexes provides the following correlation of guest structure with binding. The interior surface of compact 9 in $9 \cdot CHCl_3$ makes contact with more of the surface of $CHCl_3$ than any other guest without disturbing the rim-to-rim contacts of the host, or placing constraints on rotations of host with respect to guest. In $9 \cdot (CH_3)_3COH$, the location of the OH group is limited to the equatorial region of the sphere. A model of $9 \cdot CH_2Cl_2$ resembles a toy rattle, as the guest surface is substantially less than the inner surface of the host. The shape of propylene oxide provides even fewer contact points between host and guest in 9 -propylene oxide. Cubane is too large to fit into the cavity of the compact form of 9, so some rim-to-rim contacts must be lost in 9 -cubane that are present in free 9. Furthermore, a cube containing four protruding hydrogens fitted into a sphere provides little shared surface area. The tablet shape and large circumference of benzene limits its location to the equatorial region (1H NMR), with its plane perpendicular to the C_3 axis of the host. The host has to open somewhat to accommodate benzene, losing some rim-to-rim contacts, and providing for little shared surface area for host and guest, which accounts for the low binding¹². $CHCl_2CHCl_2$ shares a larger surface area with the host, but most of the rim-to-rim contacts are lost, accounting for the relatively low stability of $9 \cdot CHCl_2CHCl_2$. I conclude that the rim-to-rim contacts account for a substantial fraction (perhaps half) of the total binding of 9 to $CHCl_3$ in $9 \cdot CHCl_3$ (ref. 12).

In earlier work, Collet *et al.*¹³ reported the syntheses from 6 of cryptophanes 10 and 11, the former in its enantiomeric form. Complex 11 $\cdot CHCl_3$ also exists in its most compact, twisted form (60° rotation, crystal structure determination), as illustrated in formula 11. From the ΔH and ΔS values reported for 11 binding its most complementary guest, $CHCl_3$ ($CDCl_2CDCl_2$ as solvent), we can calculate that at $-20^\circ C$, $K_a = 4,300\ M^{-1}$ and $\Delta G_{253}^\circ = -4.2\ kcal\ mol^{-1}$. This should be compared with $K_a = 77,000\ M^{-1}$ for racemic 9 binding $CHCl_3$ in $(CCl_3)_2CO$ and $\Delta G_{253}^\circ = -5.7\ kcal\ mol^{-1}$ at $-20^\circ C$. A molecular model of 11 in its extended form seems to be more complementary to the solvent, $CDCl_2CDCl_2$, than 9 is to $(CCl_3)_2CO$ in its extended form. This may be the source of the difference in binding of $CHCl_3$ by 9 and 11. The compact forms of 9 and 11 in their CPK models have similar shapes and internal volumes.

Full incarceration

An extreme form of complex stability is found in carceplexes, hosts of which (carcerands) are noncollapsible molecular cells whose closed surfaces contain pores too small for guest molecules to enter or depart their inner phases without making or breaking covalent bonds. Formula 2 is a conceptual carcerand whose part structures inspired the study of hemispherical structures of the cavitands just described. I now describe the novel properties of carceplexes prepared by covalently bonding the rims of two cavitands to one another through four short spacer groups. The reactions are templated by solvent or solute molecules that in the process of shell closure become guest (prisoner) molecules encapsulated in the interior of the resulting carceplexes. This process can be pictured by resting the bottom of a spoon in a wine glass and placing a second glass rim-to-rim on the first, thereby incarcerating the spoon in a closed globe. The stems of the glasses extend upward and downward from the polar regions of the globe, and resemble the disposition of the feet, attached at the north and south polar regions, that are required to make carceplexes soluble.

Two ways of drawing the starting cavitands used for the shell closures are illustrated for general structure 12. Conventional synthetic methods led to starting materials for shell closures, which provided carceplexes 13-G (ref. 14) and 14-G (ref. 15) (G is solvent or solute molecules that become guests or prisoners in the product). The crystal structure of $14 \cdot (CH_3)_2NCOCH_3$ was determined, and a side-stereo view is shown. An interesting feature of the complex is that the northern hemisphere is rotated relative to the southern hemisphere by 25° around the long C_4 axis of the host. This introduces a chiral element into the complex, the host possessing nearly D_4 symmetry with four short C_2 axes. The guest is ordered in the crystal in the sense that appropriate guests occupy the same plane, but is disordered in the sense that the Y-shaped $(CH_3)_2N$ - and $-COCH_3$ groups are randomly interchanged in different carceplex molecules.

The yields of the carceplexes in the shell closures leading to

13-G varied between 20 and 32%, depending on the solvent. Carceplexes 13-2CH₃OH, 13-2CH₃CN, 13-CH₃CH₂OH, 13-CH₃COCH₂CH₃, 13-CH₃CH₂COCH₂CH₃ and 13-(CH₃)₂NCHO were isolated and fully characterized. No empty host (carcerand) was produced, nor was any 13-C₆H₆, when C₆H₆ was used as a cosolvent in preparing 13-2CH₃OH and 13-CH₃CH₂OH, although CPK molecular models of 13-C₆H₆ are easily assembled. The shell closures therefore show high structural recognition. The absence of empty carcerand is explained by the high entropic and desolvation costs of generating a vacuum of considerable size in the area of the reacting groups¹⁴.

Molecular models (CPK) of these carceplexes show the cavity to be elongated in the north-south dimension, narrowing towards the poles and widening at the equator, like an American football. The only holes remotely large enough to allow guests to escape are centred at the two poles, around which are the two sets of four appended groups whose conformational mobility makes the complexes soluble. When heated to 110 °C in toluene for 3 days, 13-2CH₃CN loses a guest to give 13-CH₃CN + CH₃CN. When heated to 215 °C in 1,2,4-trichlorobenzene for 5 days, 13-CH₃CN is stable. I attribute the thermal instability of 13-2CH₃CN to a 'billiard-ball' effect, in which high-energy collisions between guests aligned along the north-south axis result in expulsion of one guest through one of the polar holes. Collisions of a single guest with the sides of the container do not properly aim the rebound for the guest to escape. When 13-2CH₃OH was heated for 5 days in toluene at 110 °C, no expulsion occurred. The stability of 13-2CH₃OH is attributed to the hydrogen bonding of the two guests to one another, as well as to the four equatorial sulphide groups¹⁴.

The symmetry properties of the ¹H NMR spectra of complexes 13-G showed that in 13-2CH₃CN, 13-CH₃CN, 13-2CH₃OH, 13-CH₃CH₂OH, and 13-(CH₃)₂NCHO, the guests are rotating rapidly on the spectral (and human) timescale around all of the host's axes at available temperatures. In the ¹H NMR spectrum of 13-CH₃COCH₂CH₃, the north and south protons of the host were different, but the east-west protons gave the same chemical shifts. Clearly, the long polar axis of the host and the long axis of the guest are aligned. The guest rotates rapidly about this long axis, but is not able to rotate end to end. The same constraints undoubtedly apply to the guest of 13-CH₃CH₂COCH₂CH₃, but its like-ended structure provides the same chemical shifts for the corresponding north-south protons of the host¹⁴.

In the shell closures leading to 14-G, eight covalent bonds were made in the same step leading to 14-(CH₃)₂SO (61% yield), 14-(CH₃)₂NCOCH₃ (54%), and 14-(CH₃)₂NCHO (49%), depending on the solvent. These yields are remarkably high. That these reactions are solvent-templated was shown by the fact that when CH₂(CH₂CH₂)₂NCHO was the solvent (too large for the cavity of 14), no carceplex was produced. When 0.5 mol % of (CH₃)₂NCOCH₃ in CH₂(CH₂CH₂)₂NCHO was used as solvent, a 10% yield of 14-(CH₃)₂NCOCH₃ was realized. When a 1:1 molar ratio of (CH₃)₂NCOCH₃ and (CH₃)₂NCHO was used, a 5:1 ratio of 14-(CH₃)₂NCOCH₃ to 14-(CH₃)₂NCHO was obtained in a collective 27% yield. These shell closures provide further examples of structural recognition of incipient host for guest¹⁵.

Temperature-dependent ¹H NMR spectral studies of host in CDCl₃ and in C₆D₅NO₂ established that (CH₃)₂NCHO in 14-(CH₃)₂NCHO can rotate around all its axes rapidly on the spectral timescale at all available temperatures, and that (CH₃)₂NCOCH₃ in 14-(CH₃)₂NCOCH₃ can rotate only about its long north-south axis. Above 2 °C, the (CH₃)₂SO guest in 14-(CH₃)₂SO rotates rapidly on the ¹H NMR timescale around all axes, but rotations about the short equatorial axes were slow below 2 °C (ref. 15).

Three pieces of evidence indicate that incarcerated guests can communicate with molecules outside the cages: (1) an ordered

coplanar arrangement of guests is observed in the crystal structure of 14-(CH₃)₂NCOCH₃; (2) carcerands containing a common host but different guests are easily separated chromatographically; and (3) the guest's proton signals (external reference) in the ¹H NMR spectra of (CH₃)₂NCOCH₃ in C₆D₅NO₂ or in CDCl₃ as solvent differed in chemical shift by Δδ values between -0.04 and -0.10 p.p.m., whereas Δδ values for 14-(CH₃)₂NCOCH₃ dissolved in these two solvents were between +0.24 and +0.33 p.p.m. We interpret these effects in terms of the guest's dipoles inducing dipolar changes in the host's shell, which in turn interact differently with the environments of the carceplex molecules¹⁵.

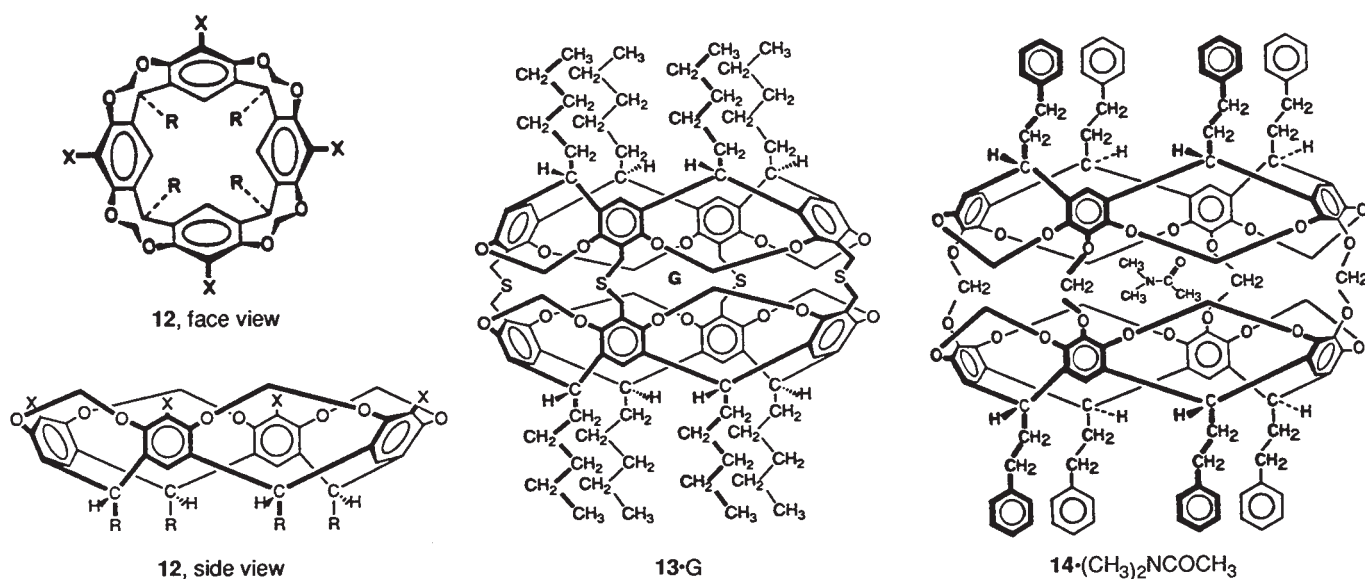
The C-N rotational barriers of (CH₃)₂N-COCH₃ and (CH₃)₂N-CHO were determined by dynamic ¹H NMR experiments in C₆D₅NO₂ and compared to the rotational barriers observed in their respective carceplexes dissolved in the same solvent. The barrier to rotation of incarcerated (CH₃)₂NCOCH₃ was a little over 1 kcal mol⁻¹ higher than that of free amide. Molecular models of 14-(CH₃)₂NCOCH₃ show that the guest is compressed against the sides of its container, even more tightly than when it is intercalated into a solvent. In contrast, the barrier for incarcerated (CH₃)₂NCHO was ~2 kcal mol⁻¹ lower than that for free amide. Molecular models of 14-(CH₃)₂NCHO show the guest to be loosely held by its container. Generally, amide barriers to rotation decrease with phase changes in this order: polar solvents > nonpolar solvents > gas phase. The inner phases of carceplexes are unique in the sense that they offer different blends of vacuum and space occupation, depending on the relative dimensions of the container interiors and of their guest occupants¹⁵.

Hemicarcerands and hemicarceplexes

Hemicarcerands are rigidly hollow molecules whose interiors are of molecular dimensions and whose shells contain portals through which guests can pass in either direction, but with high enough steric barriers to allow easy isolation and study of their complexes at ordinary working temperatures. That part of the free energy of activation for decomplexation due to steric effects we refer to as 'constrictive' binding. Generally, hemicarceplexes can be prepared by shell closures, isolated, purified and characterized. When heated to 100–220 °C in high-boiling solvents too large to enter the interior of the hemicarcerand, the complex dissociates to give free hemicarcerand and guest. By heating hemicarcerands to high temperatures in the presence of large excesses of potential guests, new hemicarceplexes can be formed, the encapsulation being driven in part by the mass law.

One type of hemicarcerand (15) was prepared by shell closures involving only three of the four hemispheric bridging groups found in carcerand 14. The shell closures run in (CH₃)₂SO as solvent gave 15-(CH₃)₂SO (51%), in (CH₃)₂NCOCH₃ gave 15-(CH₃)₂NCOCH₃ (42%), and in (CH₃)₂NCHO gave 15-(CH₃)₂NCHO (20%). The three hemicarceplexes were fully characterized, and a crystal structure of 15-(CH₃)₂NCHO·2CH₃CN·2CHCl₃ was determined. The two CH₃CN molecules are packed between the two sets of (C₆H₅CH₂CH₂)₄ feet with the N pointing inwards. Each packet is capped with a CHCl₃ molecule. The (CH₃)₂NCHO guest is ordered and oriented with its C=O bond pointing toward the portal (the nonbridged equatorial position). The top hemisphere of 15-(CH₃)₂NCHO·2CH₃CN·2CHCl₃ is rotated around its polar axis by ~13° relative to the bottom, which allows the two sets of intrahemispheric (OCH₂O)₄ groups to mesh like gears around the equatorial region of the complex. The hemicarceplex has C₂ symmetry, the C₂ axis passing through the O and N atoms of the guest¹⁶.

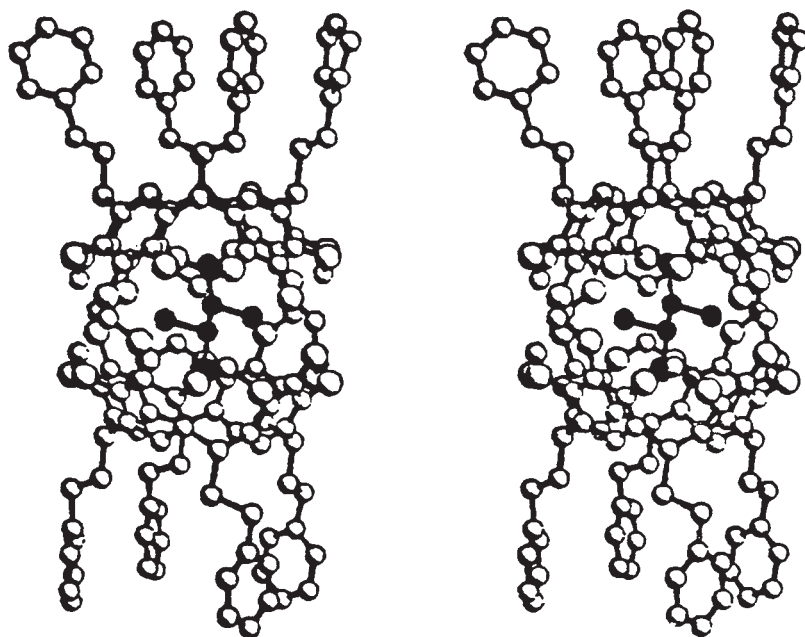
By heating 15-G in the large solvent, 1,2,4-Cl₃C₆H₃, the three complexes liberated their guests with different half-lives as follows: 15-(CH₃)₂SO at 195 °C, *t*_{1/2} = 24 hours; 15-(CH₃)₂NCOCH₃ at 140 °C, *t*_{1/2} = 34 hours; and 15-(CH₃)₂NCHO at 140 °C, *t*_{1/2} = 14 hours. In each case, free



carcerand **15** was produced essentially quantitatively and was characterized. In stretched CPK molecular models of **15**, the portal is roughly slot-shaped, as are the cross-sections of the two amide guests, allowing these guests to pass through the portal more easily than the more spherical guest, (CH₃)₂SO. Empty host **15** is incapable of undergoing any conformational change to fill its own cavity. Thus, dissolution of **15** in CDCl₃, a solvent too large to enter the host, is the equivalent of dissolving holes in a liquid. Organic liquids are only ~30% empty space¹⁷, present as small volumes between molecules that make inefficient contact with one another because of their shapes. The accumulation of many small volumes into a single large volume, as in the hole in **15**, is entropically unfavourable. Filling that hole with small dissolved molecules is favourable both because of the entropy of dilution of the small molecules and because of the entropy of dilution that accompanies turning a large empty space into many small empty spaces normally between molecules in the liquid state. Therefore, ubiquitous small molecules such as N₂, O₂, H₂O and CO₂ that are dissolved in CDCl₃ containing **15** should reversibly enter the cavity and become guests. This phenomenon was observed by differences in ¹H NMR spectra when solutions of **15** were saturated with these small compounds. The *K_a* value at 22 °C for **15**·N₂ is ~180 M⁻¹, and that for **15**·O₂ is ~44 M⁻¹ (ref. 16). The diamagnetic character of **15**·O₂ caused the signals of protons next to the guest in **15**·O₂ to shift and to broaden greatly¹⁸.

In CPK models of **15**, the portal can be widened enough to accept a spherical model of xenon of diameter equivalent to 4.4 Å. At 22 °C, the half-life for complete complexation of a xenon-saturated solution of **15** in CDCl₃ at 22 °C was ~90 min (¹H NMR measurements). The complex, **15**·Xe, was isolated and thoroughly characterized. Its decomplexation at 25 °C had a half-life *t*_{1/2} = 47 h, and its *K_a* ≈ 200 M⁻¹ at 22 °C and -Δ*G*° ~ 3 kcal mol⁻¹ (ref. 16).

Other complexes that formed at 25 °C in guest or guest-CHCl₃ as solvent were **15**·CH₃CN, **15**·2CH₃CN, **15**·CH₂Cl₂, **15**·CH₂Br₂ and **15**·CS₂, all of which except **15**·2CH₃CN were isolated pure and characterized. The ¹H NMR spectrum of **15**·2CH₃CN suggests that the two guests are arranged with their two C≡N groups each occupying the polar regions, and with each of their

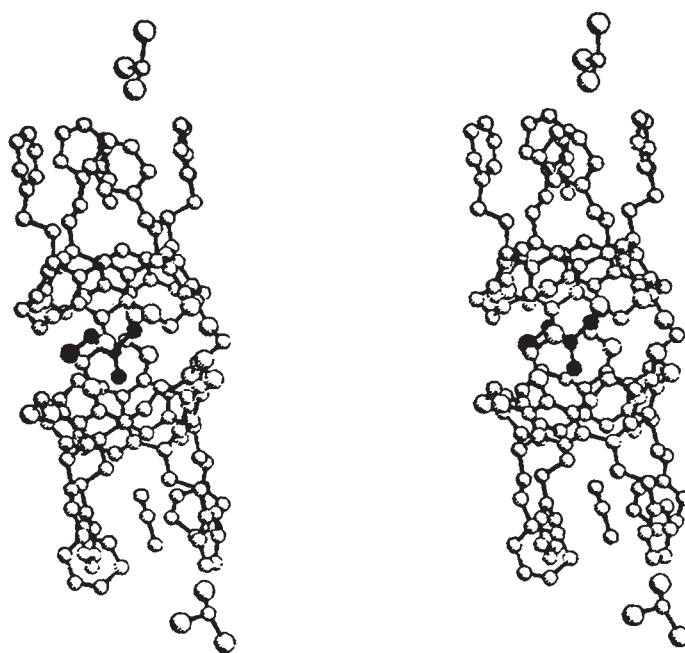
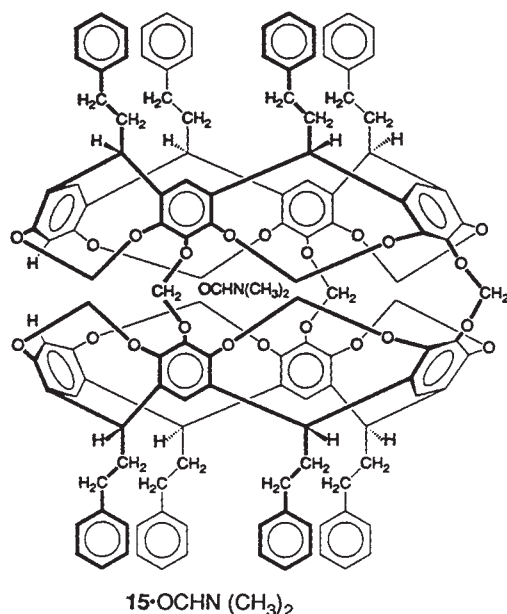


Crystal structure side stereoview
14·(CH₃)₂NCOCH₃ with guest atoms darkened

two CH₃ groups in the wider equatorial part of the cavity. This complex tended to produce **15**·CH₃CN and free CH₃CN unless it was kept in the presence of free CH₃CN. Treatment of a 0.5 mM solution of **15**·CH₂Br₂ in dry (CH₂)₄O with 300 equivalents of butyllithium for 1 min at 25 °C had no effect on the complex, whereas free CH₂Br₂ reacts instantly with this reagent. The shell can act as a shield which protects its guests from reactants in the bulk medium¹⁶.

Larger guests became incarcerated only at elevated temperatures, so **15**·pyridine, **15**·(CH₂)₄O and **15**·C₆H₆ were prepared by heating **15** in the guest-solvent. Larger guests such as CH₃C₆H₅ could not be incarcerated in **15**. The amines (CH₃CH₂)₂NH and CH₃(CH₂)₃NH₂ were introduced into **15** by heating them in C₆H₅Cl for several hours.

Incarcerated amines are much weaker bases than free amines dissolved in CDCl₃. A large excess of CF₃CO₂D failed to give **15**-pyridinium ion under conditions in which free pyridine in CDCl₃ was quantitatively converted to pyridinium. The host poorly solvates charge and inhibits the formation of contact ion

Side stereoview of 15-OCHN(CH₃)₂ · 2CH₃CN · 2CHCl₃

pairs. When 15-(CH₃CH₂)₂NH in CDCl₃ was treated with 100 equivalents of CF₃CO₂D, the complex instantaneously dissociated (because of guest protonation). Although 100 equivalents of CH₃CO₂D failed to dissociate the complex, it did cause deuteration of the guest's amino group. This isotope exchange is facilitated by the closeness of the NH group to the equatorial portal (CPK models). Although the presence of 100 equivalents of CF₃CO₂D in CDCl₃ greatly accelerated decomplexation of 15-CH₃(CH₂)₃NH₂, the estimated half-life for decomplexation of protonated complex was still 22 min. Titration experiments of 15-CH₃(CH₂)₃NH₂ with CF₃CO₂D showed the coexistence in CDCl₃ of comparable amounts of 15-CH₃(CH₂)₄ND₂⁺ and 15-CH₃(CH₂)₄ND₃⁺, which indicates that the pK_a of bound butylammonium ion is not far from that of CF₃CO₂D in CHCl₃. The inner phase of 15-G, being a blend of guest, container sides and vacuum, tends to destabilize charged species much more than the outer CDCl₃ solution phase¹⁶.

Beautifully preorganized hemicarcerand **16** was synthesized by condensing two bowl-shaped tetra-aldehydes with 1,3-(H₂N)₂C₆H₄ in 45% yield. Eight double bonds were formed leading to octamine, all of whose carbon-nitrogen double bonds were of the anti-configuration. In CPK models, this compound contains four large portals in the equatorial region with the same polar caps as those present in **15**. Free host was easy to isolate, as only large compact molecules can be incarcerated in **16**. The latter were introduced either by melting a host- and guest-mixture of solids for many hours at temperatures of 80–168 °C, or by heating them in concentrated solutions in A, a solvent whose molecules are too large to enter **16**. The hemicarceplexes formed were easily purified and characterized. Guests that formed isolable hemicarceplexes with **16** are referred to by the letters B–U. These complexes are stable at ordinary temperatures. From their ¹H NMR spectra, it is clear that their stability is due to constrictive binding. For example, the CH₃ and (CH₃)₂CH groups of menthol (F) and the 2,3,6,7-protons of anthracene (J) are held by the polar caps of the host. The half-lives for guest liberation from **13** of the complexes in CDCl₂, CDCl₂ or CDCl₃ were measured, and ranged from a low of 3.2 h at 25 °C for CCl₂=CCl–CCl=CCl₂ (B) to a high of 19.6 h for ferrocene (T) at 112 °C. The free energies of activation for liberating adamantane (N) and ruthenocene (U) from their

complexes were 19 and 28 kcal mol^{–1}, respectively. Although CPK molecular models of **16** could be assembled around [3.3]-paracyclophane, this compound did not enter **16** at high temperatures. In this series, [2.2]paracyclophane, C₁₆H₁₆ (M) is the largest guest that entered **16** (ref. 19).

Structural recognition in hemicarceplexes

The first thermodynamic stereoselectivity for hemicarceplexes binding chiral guests, reported by Collet *et al.*, involved an enantiomer of the cyclotrimeratrylene-based cryptophane host **10** complexing CHFCIBr. These authors observed at 53 °C a difference in thermodynamic stability of ΔΔG° = 260 cal mol^{–1} between their two diastereomeric complexes²⁰.

In our studies²¹, host **17** was prepared (12% yield) by shell closure of bowl-shaped tetrol with the optically pure enantiomers of dibromide, (*R*)-**18** and (*S*)-**18**. In CPK models of **17**, the size of the four chiral equatorial portals and the chiral cavity varies with the dihedral angle between the two naphthylene rings. When this dihedral angle is minimal, the two hemispheric rims touch one another in many places. When the angle is maximal, both the portals and the cavity are large but the rim-to-rim contacts are lost²².

On heating 17·CHCl₃ in the appropriate guest-solvents, guest exchange occurred to produce 17-*p*-CH₃C₆H₄CH₃ (100 °C), 17-CH₃CHICH₂CH₃ (70 °C), 17-CH₃CHOHCH₂CH₃ (90 °C), and 17-BrCH₂CH(CH₃)₂ (90 °C). The half-lives (*t*_{1/2}, hours) for guest departure (exchange) when dissolved in CDCl₃ at 23 °C for these hemicarceplexes were as follows: 17-*p*-CH₃C₆H₄CH₃, 4; 17-CH₃CHICH₂CH₃, ~50,000; 17-CH₃CHOHCH₂CH₃, 2; 17-BrCH₂CH(CH₃)₂, 0.33. The rates for decomplexation decreased by a factor of more than 10⁴ with the small change in guest structure from CH₃CHOHCH₂CH₃ to CH₃CHICH₂CH₃. An even more striking example of structural recognition in complexation-decomplexation is the failure of 17·CHCl₃ to produce 17-CH₃CH₂C₆H₅ when heated for 20 h at 140 °C, whereas 17-*p*-CH₃C₆H₄CH₃ readily formed at 100 °C in 18 h. This result correlates with the fact that CPK models of 17-*p*-CH₃C₆H₄CH₃ are readily made, whereas those of 17-CH₃CH₂C₆H₅ cannot be assembled.²²

For study of chiral recognition in guest release, one-to-one hemicarceplexes were prepared by heating in the dark (*R*₄)-**17**

or (*S*₄)-**17** at 100 °C dissolved in chiral guests. From enantiomerically pure (*S*)-BrCH₂CH(CH₃)-CH₂CH₃ were prepared (*R*₄)-**17**·(*S*)-BrCH₂CH(CH₃)-CH₂CH₃ and (*S*₄)-**17**·(*R*)-BrCH₂CH(CH₃)-CH₂CH₃, whose first-order rate constants for guest exchange in CDCl₃ as solvent at 23 °C were measured²² to provide $k_{R_4S}/k_{S_4S} = 7$.

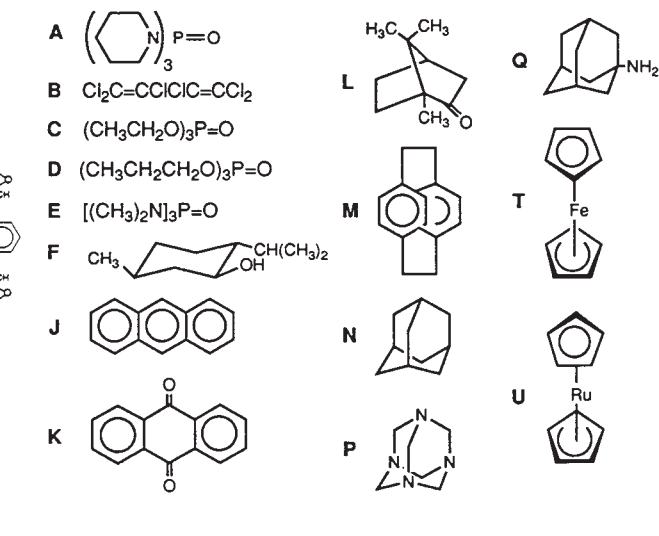
From (*S*₄)-**17**·CHCl₃ and racemic BrCH₂CH₂CHBrCH₃ was similarly prepared a mixture of diastereomeric complexes (99% yield) in which one diastereomer dominated (¹H NMR spectral measurements) by a factor that varied in three identical runs from 1.5:1 to 2:1. Times that varied between 0.75, 1, 24 and 336 hours gave ratios between these limits, showing that equilibria were established. Thus the difference in free energies of association for the diastereomeric complexes was ~300 cal mol⁻¹ at 100 °C. In CDCl₃ at 23 °C, the ratio of relative first-order rate constants for diastereomeric guest-exchange was $k_{fast}/k_{slow} = 5$. The less thermodynamically stable isomer gave the faster rate. Similarly, from (*S*₄)-**17**·CHCl₃ and racemic BrCH₂CHBrCH₂CH₃ was prepared an equilibrated mixture of diastereomeric complexes in a ratio of 2:1. In CDCl₃ at 23 °C, the ratio of relative first-order rate constants for diastereomeric guest exchange was $k_{fast}/k_{slow} = 9$. In this case, the more stable diastereomer gave the faster rate²².

The differences in activation free energies ($\Delta\Delta G^\ddagger$) for the diastereomeric complexes dissociating (the slow step in the exchange) at 23 °C in CDCl₃ were 1.1 kcal mol⁻¹ for BrCH₂CH(CH₃)-CH₂CH₃, 1.0 kcal mol⁻¹ for BrCH₂CH₂CHBrCH₃, and 1.3 kcal mol⁻¹ for BrCH₂CHBrCH₂CH₃. The $\Delta\Delta G^\circ$ values at 100 °C for the latter diastereomers are ~300 cal mol⁻¹. Usually, $\Delta\Delta G^\circ$ values for diastereomeric complexes decrease with increasing temperature. If $\Delta\Delta G^\circ$ is assumed to have remained at ~300 cal mol⁻¹ at 23 °C, the $\Delta\Delta G^\ddagger$ value for the complexing diastereomeric transition states would be 1.6 kcal mol⁻¹ for BrCH₂CHBrCH₂CH₃ ($k_{fast}/k_{slow} \approx 15$) and 0.7 kcal mol⁻¹ for BrCH₂CH₂CHBrCH₃ ($k_{fast}/k_{slow} \approx 3$)²². These values provide a measure of chiral recognition in complexation-decomplexation and are strikingly high.

Differences in steric repulsions in the diastereomeric transition states are probably responsible for this chiral selectivity. With each of the three chiral guests examined, the host discriminates between the steric requirements for CH₃ and Br, or CH₂CH₃ and CH₂Br. The relative sizes of covalently bound CH₃ and Br calculated from their volumes and surface areas differ by only 5–10%. Much higher stereoselectivity is expected when larger steric requirements are involved²².

Stabilized cyclobutadiene in hemicarceplex

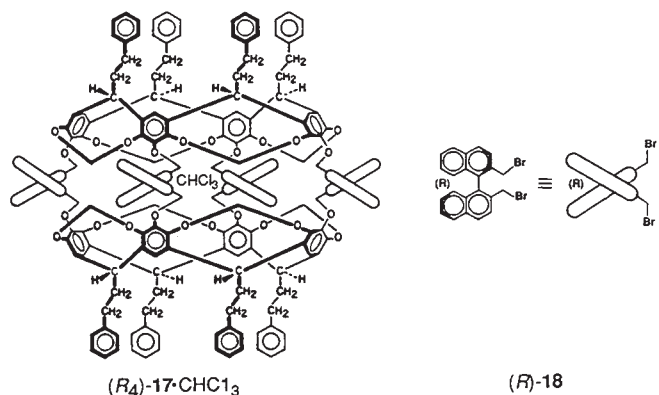
Cyclobutadiene ((CH)₄, or **18**), being the smallest carbocycle composed of conjugated double bonds, is of considerable importance to the structural theory of organic chemistry. Efforts have been made to synthesize it for over a century^{23–25}. The compound has been prepared as a fleeting intermediate by a variety of methods, but has been isolated only in an argon matrix at 8 K by the photolysis of α -pyrone (**19**)^{26,27}. Corey *et al.*^{28,29} had previously converted **19** photochemically to give lactone **20**, which when heated gave rearranged lactone **21**. Flash vacuum pyrolysis³⁰ at 600 °C converted **21** to **19**. Attempts to isolate cyclobutadiene led to their isomeric dimers (**22**), which when heated gave cyclooctatetraene (**23**)^{24,25}. But Maier *et al.*³¹

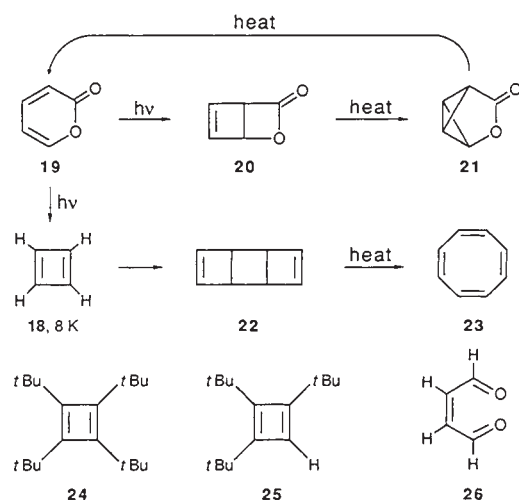


and Masamune³², respectively, prepared derivatives **24** and **25**, whose tertiary butyl groups stabilized the molecules by sterically inhibiting their reactions.

We synthesized cyclobutadiene (**18**) lodged in the inner phase of hemicarceplex **15** (ref. 33) where it is protected from dimerization^{24,25} by its surrounding shell, and from reactants too large to pass through the portal of **15** at 25 °C. Hemicarceplex **15**·**19** was prepared by heating empty **15** in 3:1 C₆H₅Cl/ α -pyrone and precipitating the cooled complex with hexane. When degassed CDCl₃ or (CD₂)₄O solutions of **15**·**19** sealed in borosilicate glass tubes were irradiated at 25 °C with a 75-W xenon arc lamp for 30 min, **15**·(CH)₄ was produced, whose ¹H NMR spectrum demonstrated the cyclobutadiene to be in its singlet ground state. The CO₂ was expelled from the shell, probably during the reaction. Continued irradiation of the tube led to acetylene, a reaction previously observed to occur²⁶ in the argon-matrix synthesis of **18**. The acetylene escaped through the portal of its hemicarceplex and was characterized. When the tube containing **15**·(CH)₄ in (CH₂)₄O was heated for 5 min at 220 °C, the cyclobutadiene escaped from its protective shell and dimerized to give **22**, which rearranged to cyclooctatetraene (**23**)^{24,25}, which was characterized. The complex **15**·(CH)₄ in CDCl₃ as solvent reacted with O₂ to give the complex of malealdehyde, **15**·**26** (ref. 33).

In the photochemical conversion of **15**·**19** to **15**·(CH)₄, light of wavelengths above 200 nm was used. Host **15** has an ultraviolet spectrum that ends abruptly at 300 nm, whereas that of α -pyrone (**19**) has end absorption that terminates at ~350 nm. When a solution of **15**·**19** in CDCl₃ was irradiated with light of >300 nm at 25 °C, **15**·**20** was produced quantitatively and characterized. When **15**·**20** as a solid was heated at 90 °C for 17 h, 80% of **15**·**21** was produced and characterized. When





heated as a solid at 140 °C for 20 h, **15-21** reverted to **15-19**. Thus the cycle of reactions **19** → **20** → **21** → **19** occurred in the inner phase of hemicarcerand **15**, bringing the number of transformations in this inner phase to six. Of these, three were photochemical (**19** → **18** → acetylene, and **19** → **20**), two were thermal (**20** → **21** → **19**, done in solid complex) and one was bimolecular (**18** + O_2 → **26**, done in dissolved complex)³³.

I believe it to be both realistic and useful to regard the inner phase of carcerands and hemicarcerands as a new phase of matter. It differs from the inside phases of clathrates or zeolites in its unique composition: one host molecule provides one discrete molecular inner phase. Experimentally, hemicarcerands and hemicarceplexes can exist in the solid, solution or even gaseous bulk phases (in mass spectrometers), and therefore their inner phases are not bulk-phase dependent. I expect that many highly reactive species containing bent acetylenic, allenic or

aromatic rings, radicals, carbenes and so on will be prepared and examined in the inner phases of appropriate hemicarcerands.

Potential uses

One can envisage possible uses for hemicarcerands as drug or pesticide delivery systems. I believe the hydrophilicity-lipophilicity balance of carceplexes can be manipulated by varying the terminating groups of the eight feet of the host. For example, cavitand **7** has $(CH_2)_5Cl$ feet which might be ultimately converted to $(CH_2)_5SO_3Na$ or $(CH_2)_5PO_3Na_2$ groups. An alternative involves building hydrophilic residues into the three or four hemisphere bridging groups. Amantidine (**Q**) is the only drug as yet incarcerated (**16·Q**).

Most medicinals, antibiotics and pesticides have relative molecular masses between 300 and 400, whereas the largest guest I have incarcerated is [2.2]paracyclophane (**16·M**), with a molecular weight of 208. I am currently addressing the problem of making hemicarcerands with larger cavities, some containing water-solubilizing groups. Particularly interesting is the possible alteration of the biological spectrum of antibiotics by changing the hydrophilicity or lipophilicity of their container compounds.

Other possibilities include incarcerating metal complexes useful in organ imaging, or radiation delivery systems that keep heavy metals from being deposited in bone. Carceplexes and hemicarceplexes can contain guests with large dipole moments; if the guests are mobile with respect to their hosts they could be aligned by electric fields. The ultraviolet and visible spectra of the guests can also be designed. Electrically controlled light switches of molecular dimensions might be designed and prepared. Another possible use is the incarceration of catalysts whose mode of action is controlled by the spatial constraints of the portals and the inner phases. New phenomena in organic chemistry breed applications. □

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The *C. elegans* genome sequencing project: a beginning

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The long-term goal of this project is the elucidation of the complete sequence of the *Caenorhabditis elegans* genome. During the first year methods have been developed and a strategy implemented that is amenable to large-scale sequencing. The three cosmids sequenced in this initial phase are surprisingly rich in genes, many of which have mammalian homologues.

THE realization that the human genome can be sequenced in its entirety has stimulated great interest in genome analysis^{1,2} and efforts have already begun to construct genetic and physical maps³ and to improve DNA sequencing methods⁴. In pursuit of this great enterprise, it will be necessary to sequence and analyse the smaller genomes of experimentally tractable organisms which will serve as pilot systems for evaluating technology and also provide information essential for interpreting the human sequence. The genomes of single-celled organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* will reveal features peculiar to the basic functions shared by all living things. Analysis of simple animal genomes intermediate in size and complexity between those of yeast and man will help us to understand the more complex features of mammals.

The genome of the small nematode *C. elegans* is a good candidate for complete sequence analysis⁵. This organism has been used to investigate animal development and behaviour^{6,7}. Its small size and short generation time facilitate genetic analysis, and more than 900 loci have now been identified through mutations⁸. Each animal develops with essentially the same pattern of cell divisions, and this entire pattern is known⁹⁻¹¹. The anatomy is simple, with only 959 somatic cells, and the ultrastructure established—for example, the complete connectivity of its 302 neurons has been determined¹².

Many genes required for normal development and behaviour are being studied. With the aid of an active transposon system¹³⁻¹⁵ and a physical map of the genome¹⁶⁻¹⁸, they can be readily cloned. Methods for transformation have been developed which allow reintroduction of engineered genes¹⁹⁻²¹. The similarity of many of these genes to those in mammals is often extensive, supporting the contention that information obtained in the nematode will be relevant to understanding the biology of man.

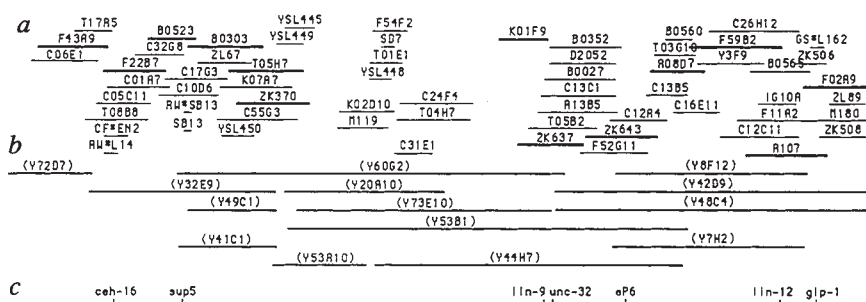
The *C. elegans* genome contains an estimated 100 megabases (10^8 bases), less than the size of an average human chromosome. Generally genes in *C. elegans* have smaller and fewer introns than their mammalian counterparts²² and the gene density is high (see below). A clonal physical map of the nematode genome is nearly complete¹⁸. A combination of cosmid and yeast artificial chromosomal (YAC) clones has been used to reconstruct more than 95 megabases (Mb) of the genome. More than 90 Mb have been positioned along the chromosomes using genetically mapped sequences and through *in situ* hybridization of cloned sequences. Fewer than 40 gaps now remain in the map and progress towards closure is proceeding steadily (A.C. *et al.*, unpublished results).

We have embarked on a project to determine the entire sequence of the nematode genome. The region where we began is the centre of chromosome III (Fig. 1). There is good cosmid coverage over most of several megabases, and the few areas lacking cosmid coverage are spanned by YACs. The region lies in the central gene-rich cluster of chromosome III, where several genes of interest have been mapped.

Strategy and methods

The physical map of *C. elegans* was constructed using cosmid and YAC clones, but directed sequencing of even cosmid clones proved impractical because of the presence of repeat sequences and problems with obtaining sufficient quantities of template DNA. Thus we began by generating random subclones from

FIG. 1 Physical map of the region where the sequencing project has begun. **a**, Selected overlapping cosmid and lambda clones are represented by the horizontal lines. The cosmids which have been sequenced and reported here are underlined in bold, as well as the additional cosmids underway at present. **b**, The overlapping YAC clones from the region bridge the segments not represented in cosmid clones above. Methods must still be developed to capture sequence efficiently from the spans presently cloned in YACs only. **c**, Six genes and markers known to lie in the regions from genetic and other studies⁵⁰. The genes *unc-32* and *sup-5* are less than 0.1 centimorgans apart on the genetic map of chromosome III. Because of the way the physical map was constructed^{16,17}, the physical distance separating these two genes can only be estimated to be more than 150 kb, yielding a ratio of more than



1.5 Mb per centimorgan. This metric is typical of the central chromosomal clusters in *C. elegans*.

sheared, sized DNA^{23,24}. Libraries of small inserts (1–3 kilobases (kb)) were convenient and larger insert libraries (6–9 kb) were useful for establishing continuity in gap closure.

To collect the sequence data, we relied largely on two fluorescent-based sequence-gel readers, the Applied Biosystems ABI 373A and the Pharmacia ALF, which provide data directly in machine-readable form. All data were transferred to Unix-based Sun workstations. A display editor was developed to allow rapid clipping of the vector from the 5' end and unreliable sequence from the 3' end²⁵.

In the first phase of data generation, single reads of about 400 base pairs (bp) were taken from one end of the random clones using the ABI 373 instrument and *Taq* polymerase with a cycle sequencing protocol that reduced the amount of template necessary^{26,27}. After 100–350 reads were obtained (the optimal number will ultimately depend on relative costs and has not been established; Table 1) and assembled into contigs using Staden's assembly program²⁸, the project switched to a directed phase for closure and finishing. As a preliminary directed step, a reverse read was sometimes obtained from the opposite end of selected inserts to help establish linkage, double stranding and gap closure. Further directed sequencing required custom oligonucleotide primers, whose selection was aided by OSP (for oligonucleotide selection program)²⁹. For technical reasons, the Pharmacia ALF was more convenient for reads from custom primers³⁰. After gap closure and double stranding, in some cases further reads had to be taken, often with different chemistries^{31,32}, to resolve ambiguities. Final editing, and indeed editing throughout the project, was assisted by the ability to recall the original trace data for any region of concern from the editor program. Further details of our sequencing strategy will be published elsewhere^{33,34}.

Once the final sequence was obtained, the databases were searched for similarities using the algorithm BLAST³⁵. In addition, we have started to interpret the sequence directly. The program GENEFINDER (P.G. and L.H., unpublished) uses a statistically rigorous treatment of likelihoods to find possible genes. Other features, such as repeated sequences, are also being examined. The annotated sequences have been submitted to Genbank and EMBL databases. To present the sequences in

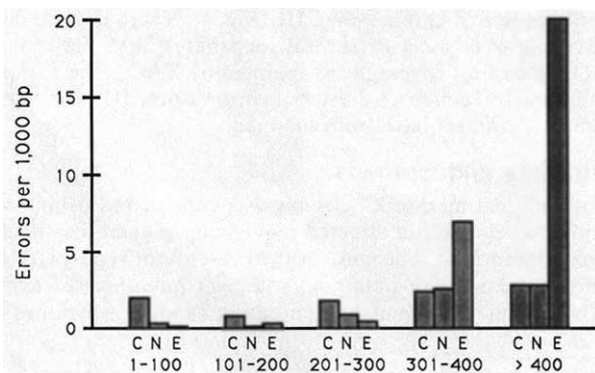


FIG. 2 The types of errors found when comparing the initial machine reads with the final edited sequence. The errors are broken down into the following categories: errors due to either the enzyme or the gel, resulting in compressions or stops (C) and errors due to the software, where there was either no call made (N, nocall) or an error made in base calling (E). In the last case the software has either inserted an extra base (an overcall), failed to recognize a base (an undercall) or simply called the wrong base (a miscall). Nocalls are less troublesome than an error in base calling, and nocalls accounted for most of the software failures in the first 300 bases of the reads. Software errors, particularly base calling errors, predominated above 300 bases. The majority of these errors were overcalls (87), as opposed to undercalls (20) or miscalls (16). The data are taken from 89 reads of single-stranded templates, with average length of 427 bases (Fig. 1), done on cosmid F59B2, for which changes in our data-handling software made the analysis easier. The data quality, however, should be similar in the cosmid sequences presented here.

TABLE 1 Sequencing strategies and statistics

Cosmid clone	B0303	ZK637	ZK643
Insert DNA size (bp)	41,071	40,699	39,528
Random subclone libraries size range:	5–6 kb	9–14 kb	1–2 kb, 6–9 kb, 9–14 kb
cloning vector:	pUC118 ⁴⁷	pBS ⁴⁸	M13mp18 ⁴⁹ , pEMBL9, pBS
Random sequencing method	ds, ABI	ds, ABI; ss, ³² P	ss, ABI
Number of random subclones	197	102	360
Number of reverse primer readings	119	70	0
Closure method	ss/ds, ALF	ds, ³² P	ss, ALF
Oligonucleotide primers required	102	417	100
Total number of readings	440	589	496
Average bp per read (vector sequence removed)	415	339	390
Total bp read (for assembly)	171,375	184,000	186,437
Final sequence redundancy	3.6	3.4	4.3

Abbreviations: ss, single-stranded template; ds, double-stranded template; ABI, Applied Biosystems Inc. 373A sequence-gel reader; ALF, Pharmacia ALF sequence-gel reader.

the context of our knowledge about the worm, we developed a *C. elegans* database, ACEDB, which holds not only the available sequences for the nematode, but also physical and genetic map information, along with reference lists and strain information (R.D. and J.T.-M., unpublished).

Sequences

Three cosmids have so far been completed (sequences submitted to the EMBL and Genbank databases) during the development of our strategy, each with method variations to improve efficiency (Table 1). The first cosmid, ZK637, was sequenced using a minimum of random clones and radioactively labelled primers for walking. The number of primers required proved costly, and the use of films to collect data made editing difficult. ZK643 was partly sequenced using restriction enzyme partial digestion for the random clone production: although subclone recovery was high, these clones were biased in their representation. B0303 DNA was sheared to produce the subclones and, as for ZK637, reverse primer sequencing was used to establish linkage before beginning directed sequencing. Closure for both ZK643 and B0303 was done using directly labelled primers on the Pharmacia ALF.

The overlap of the manual, radiolabelled sequence with the fluorescent shotgun reads in ZK637 amply confirms the fidelity of the fluorescent method. The accuracy of the base calling was tested by comparing a sampling of individual sequence reads with the final edited sequence (Fig. 2). Generally, the only errors found in the first 300 bases of a read could be attributed to compressions or stops due to gel or enzyme limitations. Above 300, software limitations predominate, with increasing numbers of sites at which either no base call can be made or errors in the base calling occur. The failure to call a base is not a serious problem as no false information enters the database. By far the most common error is overcalling the number of bases in a run, but undercalls and miscalls also occur; these errors are easily corrected, once attention has been drawn to them, by reference to the traces.

The accuracy of the final sequence is difficult to estimate without extensive independent tests, but several factors give us confidence that the fidelity to the true genomic sequence is high. All sequences were determined at least once on both strands, with the exception of certain long tandem repeat sequences. Regions with compressions or other unresolved conflicts were resequenced. Possible errors indicated by the similarity searches

and GENEFINDER analysis were checked. To detect major cloning artefacts, we compared the polymerase chain reaction products derived from the cosmids with both the predicted lengths and the products from genomic DNA across the sequenced regions. The walking primers often proved useful for this purpose. For one cosmid, part of the vector sequence was analysed which was derived from the random phase alone so that not every region had been sequenced on both strands; here only one base in 2,000 was at variance with the available sequence.

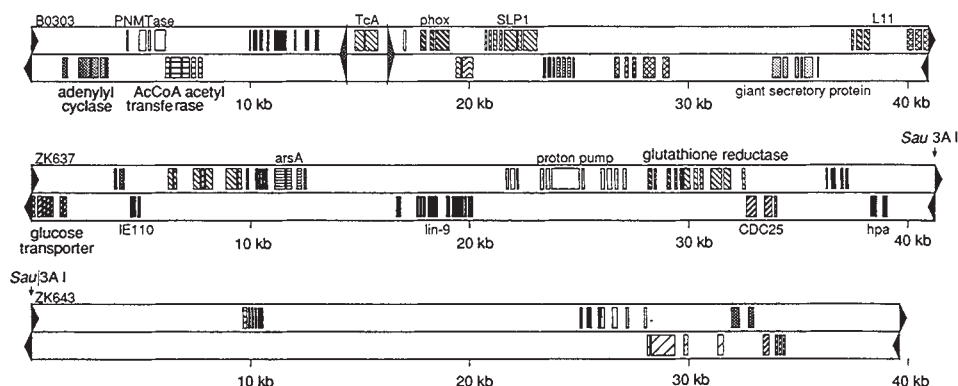
Using similarity searches and GENEFINDER to analyse these sequences, a high density of likely genes was revealed (Fig. 3). One of the most striking similarities was found between the 22–26-kb region of ZK637 and the 116K subunit (relative molecular mass 116,000) of the rat vacuolar proton pump (Fig. 4). The similarity falls into several blocks which GENEFINDER suggests are probably exons, and spans from residues 14 to 824 of the 838-amino-acid protein. The amino-terminal half is most highly conserved between the two sequences; in one stretch 99 of 139 residues (71%) are identical and in a second, 223 of 314 (71%) are identical, with no gaps introduced into the alignment. The carboxy-terminal half has less similarity but contains sequences similar to all eight postulated transmembrane domains. Other extensive similarities were found to acetyl-CoA acetyltransferase, glutathione reductase, the arsenical pump-driving ATPase, the hypothetical transposase TcA of the nematode transposon Tc1, and the host protective factor of the parasitic nematode *Trichostrongylus colubriformis*. Less extensive but still highly significant similarities (BLAST scores of >100 over one or two exons) were found with the 50S ribosomal protein L11 and the neutrophil oxidase factor. This last similarity includes a motif shared between the oxidase factor, yeast actin-binding protein ABP-1, acanthamoeba myosin IC and *sre*-related kinase³⁶. The hypothetical nematode protein has three copies of this motif. Finally, other more limited but still significant similarities of likely *C. elegans* coding regions were found with phenylethanolamine-*N*-methyltransferase, adenylyl cyclase, giant secretory protein, the yeast *CDC25* cell-cycle gene (and the *Drosophila* string homologue), glucose transporter and immediate early protein IE110 of herpes simplex virus. For adenylyl cyclase, several adjacent segments of B0303 showed similarity with a repeated motif of the cyclase sequence.

Altogether, GENEFINDER predicts a total of 33,573 bases (27%) of coding sequence in the total of 121,298 bases sequenced. For the individual cosmids the percentage of coding sequences are 37% for B0303, 33% for ZK637, and 13% for ZK643. The number of different genes represented is difficult to estimate,

as at present there are no clear rules by which to distinguish ends of genes from introns. Taking into account factors such as the distinct homologies and the spacing and strandedness of exons, we would estimate B0303 contains 14 genes, ZK637 12 genes and ZK643 6 genes. Transcript analysis will be required to test these predictions. Several studies have already centred on genes in the region. The gene *sup-5* (ref. 37) had been mapped close to the end of B0303; indeed, the B0303 sequence overlaps with the 4.2-kb region previously sequenced around the transfer RNA gene *sup-5* (K. Kondo and R.W., unpublished results). The genes *lin-9* (ref. 38) and *unc-32* (ref. 6) were known by transformation rescue to lie within ZK637 before sequencing began. The gene *lin-9* has been shown to correspond to the predicted gene on the bottom strand of ZK637 in the region 20.2–16.6, and the complementary DNA sequence used to refine the predictions of the GENEFINDER analysis (G. Beitel and R. Horvitz, personal communication). From its genetic position, *unc-32* is likely to be one of the genes immediately to the right of *lin-9*.

The sequences have also been scanned for repeats. Large, almost perfect inverted repeats (465/468) flank the region of B0303 near the 15-kb point (Fig. 3) that shares similarity with the TcA transposase; subsequent analysis revealed this region to represent a copy of the Tc1-related transposon Tc3 (D. Schneider, J. Collins and P. Anderson, personal communication). Examples of short tandemly repeated sequences include 21 copies of a 59-bp motif at 35 kb in ZK643 and two segments (11 and 13 copies) of an 11-bp motif inverted with respect to one another at 13.3 and 13.7 kb in ZK637. A larger duplicated segment is present spanning the join of ZK637 and ZK643, where blocks of 99, 305 and 789 bp spread over 1.6 kb are almost exactly duplicated 4 kb away. A 500-bp region near 7 kb in ZK643 shares several fragments with strong homology (>90%) to an 800-bp region near 32 kb in B0303, the order and orientation of the fragments being different in the two cases. A 1-kb region near 38-kb in ZK643 contains large stretches of the nucleotide motif NGG tandemly repeated; the same motif is found at the fragile X site^{39–41}. Some 20 copies of a 94-bp consensus sequence were found dispersed throughout the three cosmids, many in inverted pairs separated by up to 130 bp. Some copies showed a good match to the consensus (80–90% identity), whereas others diverged strongly or were incomplete. A search of Genbank showed the sequence to be specific to *C. elegans* and present in four copies in other entries. Although they are relatively few compared with those in mammalian DNA and are confined to local regions, these repeats would make impossible a walking strategy that relied primarily on cosmid templates.

FIG. 3 The genes in the cosmids sequenced, as determined through homology searches, GENEFINDER analysis, and available cDNA sequences. The exons of predicted genes are indicated by shaded blocks, with different shading patterns indicating distinct genes. Those shown above centre for each cosmid are encoded by the top strand and those below by the bottom strand. The genes with significant similarities to genes in the databases are indicated by the name of the most similar sequence (see Fig. 4 for details). Also shown is the position of the *lin-9* gene, as determined from cDNA sequence (G. Beitel and R. Horvitz, personal communication). ZK637 and ZK643 overlap by the single *Sau*3A1 site indicated. The inverted repeats flanking the TcA homologue are indicated by shaded triangles. The region at 25.1 to 28.1 kb of ZK643 contains two predicted genes that overlap by 52 bases but use distinct



reading frames in the overlapped segment. This is indicated in the diagram by different shading within the same block, but has not been confirmed by experiment. PNMTase, phenylethanolamine-*N*-methyltransferase; phox: neutrophil oxidase factor.

Discussion

The first three cosmids of the nematode sequencing project are complete. They represent only 0.1% of the total genome, but the methods developed have the potential to be scaled up effectively to production level. We have used two commercially available sequencing machines, but because we are proceeding on a cosmid-by-cosmid basis we have the flexibility to change as new methods and machines are developed.

The essence of our approach is to follow a conventional 'shotgun' phase, in which an initial read is taken from each subclone using a universal primer, with a 'walking' phase, in which additional reads are taken from selected subclones by the use of selected custom primers. Each subclone is large enough to allow several walking steps to be taken. Thus the shotgun phase serves not only to provide much of the final sequence but also to map the subclones for the walking phase, and the traditional problem of closure is solved efficiently without the requirement for high redundancy. Exactly when the switch is made from random clones to walking will be dictated by the costs and convenience of the two approaches.

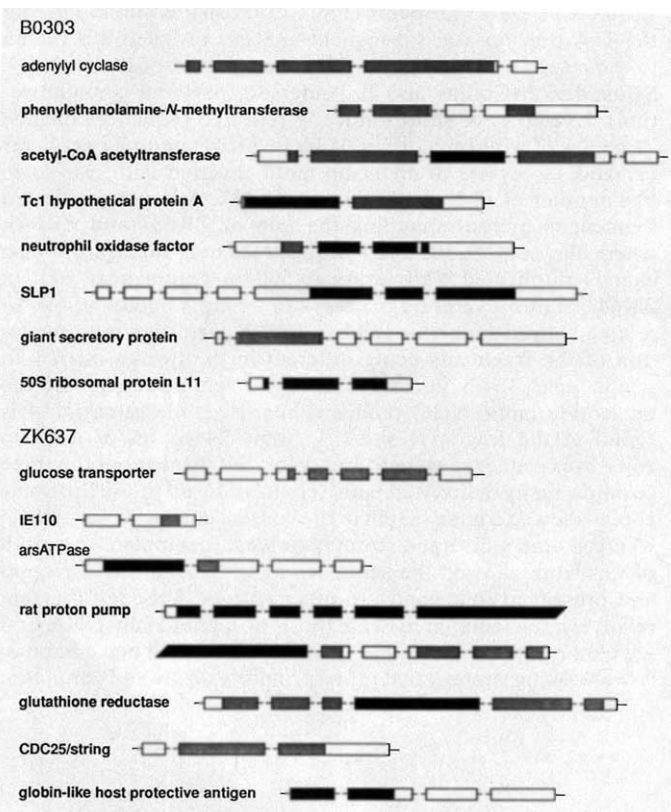


FIG. 4 The similarities of the predicted genes to previously known genes is shown in detail. The gene with the highest similarity to the proposed nematode gene is given on the left. On the right the exons of the predicted gene are indicated as blocks; homologous regions are shaded. Intervening sequences are indicated by lines and have been truncated. Hatched shading indicates regions with BLAST scores of 70 to 100, and solid shading indicates BLAST scores of greater than 100. Once similarities were detected with BLAST, the amino acids predicted from the gene were aligned with the matched gene using the FASTA algorithm⁵¹. The extent of the shading reflects the region with significant similarity determined from the FASTA output. For adenyl cyclase, the locus CYAA\$YEAST was used⁵²; for phenylethanolamine-*N*-methyl transferase, PNMT\$HUMAN^{53,54}; for acetylCoA acetyltransferase, THIL\$RAT⁵⁵; for Tc1 hypothetical protein A, CELTCB2⁵⁶; for neutrophil oxidase factor, NOF\$HUMAN⁵⁷; for SLP1, SLP1\$YEAST⁵⁸; for giant secretory protein, BARG\$CHIT⁵⁹; for 50S ribosomal protein L11, RL11\$ECOLI⁶⁰⁻⁶²; for glucose transporter, ATHSTP1⁶³; for IE110, IE11\$HSV1^{64,65}; for arsenic ATPase ARSA\$ECOLI⁶⁶; for rat proton pump, RATPRP⁶⁷; for glutathione reductase, GSHR\$ECOLI⁶⁸; for CDC25/string, CC25\$SCHPO⁶⁹; for globin-like host protective antigen, TCSHGPR⁷⁰.

The number of predicted genes in these cosmids is higher than would have been expected on the basis of genetic estimates of essential gene number⁴² and on a previous transcript analysis around the vitellogenin genes⁴³. The high gene density (one every 3–4 kb) may arise in part because the sample comes from the gene-rich cluster on chromosome III. But each chromosome contains a central gene-rich cluster, and the physical map shows that about half the genome is contained in such clusters. Extrapolating then from our (admittedly small) sample, *C. elegans* will have about 15,000 genes in the clusters alone. The other half of the DNA is not devoid of genes, but we do not yet have an accurate estimate of the gene density there. The high gene density makes genomic DNA sequencing an efficient approach to discovering complete coding sequences in *C. elegans*.

The discrepancy between genetic estimates of essential genes and our estimates of total genes based on sequence is similar to that found in other organisms. In yeast, where studies are most advanced, it is estimated that no more than one in six genes yields scorable phenotypes when function is eliminated⁴⁴. In *Drosophila*, in the region between *rosy* and *Bithorax*, more than three times as many transcripts have been found as there are mutationally defined genes, even though this is a region held to be saturated genetically^{45,46}.

More accurate prediction of genes will be aided by the accumulation of more data, providing a better basis for statistical analysis. The GENEFINDER predictions are of limited use in this regard as they necessarily reflect the biases on which they are based. Nonetheless, the success of GENEFINDER at this early stage encourages our confidence that genomic sequence is an efficient means for finding not only genes but also the other information stored in the genome. In turn, the fraction of genes that yield similarities, already about a third, will only increase and these provide immediate insights on which to design experiments to test function.

Each cosmid sequence is submitted, as it is completed, to the databases. In addition, the sequence is entered in the *C. elegans* database being developed as part of this project. Here the sequence will be available in conjunction with the physical map, the genetic map, references and strains. The ability to view the sequence in the context of much of the available knowledge about the worm should speed the assignment of function to each sequence.

The test of our strategy will come in the next two years as we scale up our efforts: we will sequence 800 kb in the first year and 2,000 kb in the second. We anticipate the cost, currently estimated at \$1 per base with current methods applied on production, will come down as we move to a mock production mode. Operator time should decrease with experience, new methods and increased automation. For example, we are experimenting with ways of preparing templates more rapidly and cheaply. Improved throughput for the sequencing machines, either by increasing the number of samples per run or by reducing the run time, is also under development by the manufacturers. Oligonucleotide costs are steadily declining with both reduced reagent usage and reduced reagent costs. Local preparation may make savings possible for some reagents in a large-scale operation. With these improvements, a reduction in costs to \$0.50 per base seems realistic. □

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LETTERS TO NATURE

Vortices on accretion disks

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EVERY rotating cosmic fluid that can be observed sufficiently closely displays either vortices or magnetic flux tubes on its surface; examples are tornadoes in the Earth's atmosphere¹, the Great Red Spot and other vortices in Jupiter's atmosphere, and sunspots. We suggest here that hot accretion disks also produce coherent objects, and that these vortices and magnetic flux tubes will cause significant dissipation and other observable physical effects. They will facilitate the escape of collimated radiation from deep within hot disks, producing spectral changes and time variability in the radiation from the disk. In the case of active galactic nuclei, modification of X-ray spectra due to the presence of vortices on accretion disks permits us to explain several observational puzzles, including short-term variability and the low degree of linear polarization.

Coherent structures are known to be common on planets and stars. As well as the examples first given, it is generally accepted that structures related to sunspots occur on other cool stars³, and there is growing evidence for spots on hot stars⁴. Laboratory experiments intended to simulate planetary atmospheres produce long-lived coherent objects under suitable conditions^{5,6}. There seems to be no natural occurrence of rotating turbulence without ordered structures embedded in it. On this basis, we must expect that accretion disks are dotted with an admixture of vortices and magnetic flux tubes.

The direct consequences of vortices and magnetic flux tubes

are most impressive when there are secondary constituents in the material of the disks. In the primitive solar nebula, the formation of dust⁷ is influenced by strong vortices in whose cores the temperature may be significantly lowered⁸. The dust can clump between the vortices on being extruded from them by centrifugal force. Likewise, in the case of hot disks, like those in active galactic nuclei (AGN), the emergent radiation will be strongly affected.

Both general considerations⁹ and elementary transfer theory¹⁰ show that vortices and magnetic flux tubes will concentrate emerging radiation into strong beams. Photons will tend to seek the low-pressure regions inside the tubes, much as hydrogen bubbles introduced into a turbulent fluid in laboratory experiments migrate into the cores of strong vortices¹¹. Once in the low-density region, the photons quickly make their way out of the disks directly from their relatively hot interiors. This radiation will be collimated and considerably harder than the radiation from a disk with no vortices, and it can provide a substantial fraction of the total luminosity of the disk. The outflowing radiation may be scattered on cooler external material before reaching the observer. From these phenomenological considerations, we can understand a number of the observed properties of AGNs.

(1) The short-term X-ray variability of AGNs is featureless in the frequency range from 10^{-3} Hz to 10^{-5} Hz. Their smooth variability spectra are described by a power-law distribution. A suggested explanation^{12,13} is that there are many small, well-separated bright spots on the disk surfaces. When, on a relativistically rotating accretion disk seen at a non-zero viewing angle, an individual spot periodically moves towards the observer, its brightness is Doppler-amplified. This produces a single line (or lines) in the variability power spectrum corresponding to the orbital frequency (and its higher harmonics). Spots distributed at a range of radii produce many lines which overlap to form a power-law spectrum^{12,13}. This empirical picture is physically natural if strong vortices or flux tubes are present.

(2) The linear polarization of most of the quiescent quasars is ~1% or lower. In the standard thin disk model, in which

opacity is dominated by electron scattering, the linear polarization is over 10% when the disk is observed nearly edge-on (at a grazing angle) and zero when the disk is observed nearly pole-on. Coleman and Shields¹⁴ have remarked that the absence of a substantial observed polarization may be due to surface roughness such as would result from the presence of many disjoint hemispherical dishes or 'craters' on the disk surface. Such surface depressions on a fluid appear where vortical or magnetic tubes protrude.

(3) Strong fluorescent emission lines of iron have been observed in some sources to be varying on very short timescales in unison with the X-ray continuum. This indicates that a large fraction of the X-radiation is reflected very close to the place of the origin of the X-rays. This cannot be explained by the standard model¹⁵ which, with its flat geometry, does not have enough surface area close to the centre on which the continuum X-ray radiation may be reflected. With the data available at present, it is not possible to decide whether the increase in reflected radiation is due to the geometry of the emission region or to anisotropy of the hard-X-ray emission process. Day *et al.*¹⁶ propose that the accretion disk is inhomogeneous, with the soft X-rays originating in the clumps and the harder X-rays coming from between the clumps. The depressions produced by vortices and flux tubes would cause the proposed beaming, and the clouds ejected by the locally enhanced radiative flux can scatter the strong X-rays.

(4) The very extended, conical regions of highly ionized gas observed in several sources suggest collimation or anisotropy of the ionizing continuum. Acosta-Pulido *et al.*¹⁷ have investigated the possibility that the collimation is produced by thick accretion disks with long narrow funnels.¹⁸ The funnels are large vortices along the axes of nearly spherical, thick disks. A mob of vortices on the surface of an almost flat thin disk may collimate the ionizing radiation equally well.

(5) The maxima of predicted continuum energy distributions emitted from homogeneous thin disks are typically in the ultraviolet, but the observed AGNs emit noticeably harder radiation.¹⁹ Because vortices permit radiation to escape from within the disk, their qualitative effects are to move the emitted continua to the soft X-ray band. This affects the interpretation of the observed thermal spectra of active galactic nuclei, and may explain especially for an observed bump in the soft X-ray band²⁰⁻²³.

Besides the issues specific to AGNs, there is the general feature that vortices on disks will produce time-dependent effects. It has been argued that discrete structures exist on the surfaces of disks and that these cause the complicated flickering of cataclysmic variables and the short-term variability of X-ray binaries²⁴. These postulated structures may be vortices on turbulent disks.

The X-ray variation of the Seyfert galaxy NGC6814 reveals a period of 12 200 s (ref. 25). The light curve can be well represented by the motion of a bright object orbiting rather near the central black hole²⁵. The possible identity of the orbiter is a long-lived coherent structure like Jupiter's Great Red Spot or the analogous persistent, stable magnetic spot complexes seen in the rapidly rotating RSA Can Ven stars (M.A.A., G. Bao, F. Fiore, A.L., E. Massaro, G. C. Perola, E.A.S. and E.S., manuscript in preparation). Such an object may be expected to produce local heating and X-rays in the manner of spots on solar-type stars.

These possible instances of gyres or magnetic flux tubes swept about in the local circulation of a disk raises the issue of when coherent objects on disks may be long-lived. The formation of such objects seems connected to the merging of vortices, a process that is a focus of current vortex research²⁷. Yet, although the existence of coherent structures on turbulent accretion disks is strongly suggested by the observations of other rotating, turbulent bodies, we are only just learning what assumptions are needed to reproduce the phenomenon of vortex formation. Two-dimensional simulations²⁷ have shown that turbulence typi-

cally forms concentrated vortices and thus differs markedly from the vision of turbulence that prevails in current accretion disk theory.

When turbulence arises in strong shear flows, the developing coherent structure concentrates the ambient vorticity into discrete objects²⁸ much as a forming star concentrates ambient matter. But the mechanism for forming such structures is not completely understood. That is why we are not able to predict that sunspots will form even as we watch the process take place. Similarly, it was not until the *Voyager* spacecraft provided closeups that the extent of vortical activity on the giant planets emerged. Indeed, simulations of Jupiter's Great Red Spot as a vortex is only about a decade old².

One difficulty in simulating long-lived jovian vortices is that the introduction of geometric conditions more like those in planetary atmospheres promotes wave generation is inimical to vortex formation^{29,30}. Simulations seem to succeed in reproducing coherent structures in planetary atmospheres and terrestrial oceans only if adequate allowance is made for the generation of vorticity fluctuations by turbulence, or if the flow is simply seeded with vorticity fluctuations which accumulate in merging vortex. The strong turbulent mixing that mollifies adverse effects³¹ in vortex formation has now been included in numerical models leading to robust vortex formation under jovian conditions³²⁻³⁴. The simulations are continuing apace³⁰ but it is unlikely that they can soon produce turbulence of the strength expected on accretion disks. Statistical mechanical arguments³⁵ have recently been adduced for the formation of objects like Jupiter's Great Red Spot, however, and we may hope to adapt these to the case of turbulent disks.

For now, we must accept the limitations of our present ability to simulate intense turbulence in any context. Such simulations as are already possible for rotational and shearing turbulence may be adapted to the study of effects peculiar to accretion disks. But we can anticipate some of the results. Radiation pressure, when the ratio of specific heats is near to 4/3, simply causes a rescaling of the equations⁸. Other effects important for accretion disks, such as strong shear and turbulence, are already included in the cited simulations. Even compressibility is effectively contained in geophysical models based on shallow-layer theory, because these involve surface density rather than density. In terms of that quantity, the equivalent two-dimensional dynamics are formally compressible and the shallow-water equations are similar to those of some astrophysical descriptions³⁶. There has also been some exploration of the hydromagnetic effects of vortices^{37,38}, and this will be important for astrophysical disks.

Simulations like those used in Solar System studies should soon be able to provide quantitative estimates of the properties of coherent structures for accretion disks, using the same kinds of assumptions that one makes for the planetary cases. In the meantime, by allowing for the presence of vortices on disks, we can immediately begin to resolve some nagging discrepancies between disk observations and theory. □

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The thermal stability of near-surface ground ice on Mars

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THE existence of subsurface water ice on Mars has been predicted in several theoretical studies^{1–5}, but there are no definitive observations of its present distribution. Geomorphic features on the surface of Mars have been widely interpreted as evidence for the presence of ground ice^{6–12}, but many of these features are found at near-equatorial latitudes, where thermal models have predicted that near-surface water ice should not be stable under present climate conditions^{3,13,14}. Here I present the results of thermal calculations which show that observed geographic variations in the thermal and reflectance properties of martian soils significantly affect subsurface temperatures. My results indicate that in certain regions, ground-ice deposits could exist much closer to the surface, and much closer to the equator, than previously thought. In the future, these deposits could be a valuable resource for human exploration¹⁵.

The thermal and reflectance properties of mid-latitude surface materials are highly non-uniform. Viking infrared thermal mapper (IRTM) observations¹⁶ show that the apparent thermal inertia of the martian surface ranges from 46 to 630 J m⁻² s^{-1/2} K⁻¹. Thermal inertia is a composite quantity defined as $I = \sqrt{k\rho c}$, where k is the thermal conductivity, ρ is the density and c is the heat capacity. As the product ρc for all geological materials varies by less than a factor of three, the thermal conductivity of martian soil must vary by more than two orders of magnitude. Of particular interest are the three large regions of low thermal inertia centred at (+20° N, 120° W), (+15° N, 330° W) and (+20° N, 215° W), which correspond geographically to the classical bright regions of Tharsis, Arabia and Elysium. Analysis of the available remote sensing data indicates that the surfaces of these regions differ markedly from those observed at close range at the Viking landing sites¹⁷, in that they are uniformly blanketed by a layer of thermally insulating, fine-grained dust, 0.1–0.2 m thick¹⁸.

Figure 1 shows the results of thermal model calculations of annual minimum, maximum and average surface temperatures as a function of latitude for 'Mars average', 'high-thermal-inertia' and 'low-thermal-inertia' soils. Annual maximum surface temperatures are higher in the southern hemisphere because

the eccentricity of the orbit of Mars brings it closer to the Sun during the southern spring and summer seasons. Soils with low thermal inertia experience higher annual maximum and lower annual minimum temperatures because the amplitudes of their daily surface temperature variations are larger. Predicted annual minimum surface temperatures are 148 K for low-thermal-inertia soils in the equatorial regions because transient, pre-dawn surface CO₂ frost forms during the coldest seasons. At higher latitudes, annual minimum surface temperatures of 148 K are due to the formation of the north and south seasonal CO₂ polar caps. Annual average surface temperatures are lower for soils with lower thermal inertia because they emit a greater proportion of their energy at infrared wavelengths near midday, when surface temperatures are near maximum. This nonlinear diurnal effect vanishes near the poles where diurnal temperature variations are absent. If the effects of subsurface heat flow are neglected, then calculated annual average surface temperatures will be the expected annual mean temperatures at depth.

Thermal model calculations of subsurface temperatures can be used to determine the expected depths of ground-ice deposits because near-surface water ice will not be stable to evaporation if temperatures exceed the local frost-point temperature for extended periods. Farmer and Doms¹³ have used observations of total column water abundances made by the Viking Mars

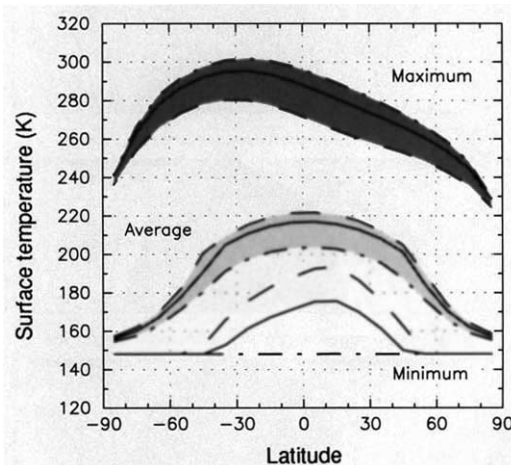


FIG. 1 Calculated annual minimum, maximum and average surface temperatures for 'Mars average' (solid line), 'high thermal inertia' (dashed) and 'low thermal inertia' (dot-dashed) soil thermal and reflectance properties. The thermal model used here is similar in concept to the original model of ref. 3, and to models that have been used subsequently^{30,31}. During each model timestep, the one-dimensional heat-diffusion equation is solved to calculate surface and subsurface temperatures, and if appropriate, surface CO₂ frost condensation or sublimation rates. Carbon dioxide frost temperatures were fixed at 148 K, CO₂ frost albedos were fixed at 0.65, and all surfaces were assumed to have unit emissivities at infrared wavelengths. A small upward heat-flow rate from the martian interior of 0.03 W m⁻² was also assumed³². The effects of atmospheric radiation on the surface energy balance were not calculated explicitly, but were accounted for by assuming that the surface is heated by the atmosphere throughout the day at a rate of 2% of the local noontime insolation, or 2% of the frost thermal emission during the polar night, whichever is greater¹⁹. The same assumption has been used to derive best-fit apparent thermal inertias and albedos from IRTM brightness temperature observations^{16,19}. The thermal inertias I and albedos A for 'Mars average', 'high-thermal-inertia' and 'low-thermal-inertia' soils are taken to be ($I=272$ J m⁻² s^{-1/2} K⁻¹, $A=0.25$), ($I=630$ J m⁻² s^{-1/2} K⁻¹, $A=0.25$) and ($I=85$ J m⁻² s^{-1/2} K⁻¹, $A=0.30$) respectively^{16,30}. The product ρc was assumed to be 1.00464 J m⁻² K⁻¹ for all cases. Soil thermal and reflectance properties were verified by using the same thermal model employed in this study to fit the available IRTM 20-μm channel brightness temperature data obtained between -20° N and +40° N over the complete diurnal cycle at a spatial resolution of ~100 km. For best-fit apparent thermal inertias between 80 and 90 J m⁻² s^{-1/2} K⁻¹, the average best-fit albedo was 0.301 with a standard deviation of 0.038.

atmospheric water detector to estimate that in the equatorial and temperature latitudes, near-surface frost-point temperatures are ~ 198 K. This estimate assumes that the observed water vapour is mixed uniformly with pressure throughout the atmospheric column. The temperatures of near-surface ground-ice deposits could exceed 198 K and still be consistent with observed column water-vapour abundances if water-vapour mixing ratios are higher near the surface than they are for the atmospheric column as a whole. For instance, a surface frost-point temperature of 203 K could be supported by 15 precipitable micrometres of total atmospheric water vapour distributed uniformly with pressure from the surface to an altitude of 8 km.

Figure 2a–c shows contours of annual maximum and minimum subsurface soil temperatures calculated for the same

three sets of model input parameters used to calculate surface temperatures in Fig. 1. The results using the 'Mars average' thermal and reflectance properties in Fig. 2a are qualitatively similar to those of previous studies^{3,13,14}, which predict that ground ice should be stable from the poles to latitudes of roughly $\pm 45^\circ$. The results in Fig. 2b show that for regions with high thermal inertia, the region of permanent ice stability should lie further below the surface and closer to the poles. The boundary is deeper because depths of penetration of diurnal and seasonal temperature waves increase as the square root of the thermal diffusivity^{19,20}. The region of permanent ice stability is further from the equator for high-inertia soil because of the nonlinear effects of thermal inertia on average temperatures discussed above. The results in Fig. 2c show that for regions of low thermal inertia and high albedo, the permafrost boundary is expected to lie closer to the surface, and closer to the equator.

An important question not considered previously is whether the formation of martian ground ice is thermally stable. To investigate, I did another set of calculations in which the thermal inertias of subsurface soil layers below the first diurnal skin depth were slowly increased as long as their temperatures remained below the frost point to simulate the formation of interstitial ice. If the temperatures of these layers ever exceeded the frost point, their thermal inertias were rapidly decreased to simulate ice sublimation. Figure 2d and e shows the results after 120 years for a global frost-point temperature of 198 K. Figure 2d assumes thermal and reflectance properties for 'Mars average' ice-free soil, like those used in Fig. 1a. Figure 2e assumes properties for 'low-thermal-inertia' ice-free soil, like those used in Fig. 2c. The results reveal that once formed, high-thermal-inertia ice deposits can be stable much closer to the surface than has been predicted by earlier models.

The stability of ground ice deposits near the surface can be explained by the tendency of the overlying ice-free surface layer

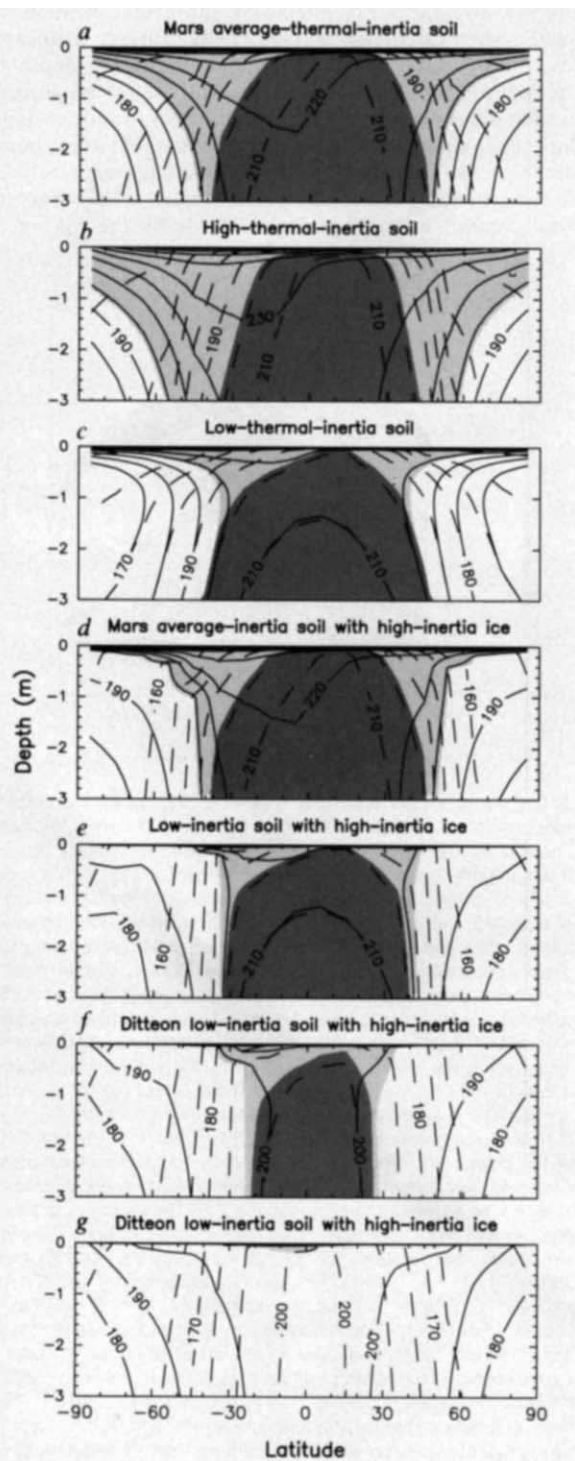


FIG. 2 Contours of model-calculated annual minimum (dashed line) and annual maximum (solid) temperatures as a function of latitude and depth. White, unshaded areas denote regions of permanent ground-ice stability, where temperatures never exceed the frost-point temperature. Lightly shaded areas denote regions of temporary ground-ice stability, where temperatures exceed the frost point during part of the year. Dark shaded areas denote the permanently frost-free regions, where temperatures always exceed the frost point. a–c, Ground-ice stability boundaries for vertically homogeneous 'Mars average', 'high-thermal-inertia', and 'low-thermal-inertia' soils assuming a frost-point temperature of 198 K. d, e, Ground-ice stability boundaries for 'Mars average' and 'low-thermal-inertia' soils overlying subsurface soil layers whose thermal properties were allowed to vary with time. At the start of the calculations, subsurface soil layers were assumed to be ice-free, and subsurface thermal properties were assumed to be uniform with depth. Each day, the thermal inertias of subsurface layers below the first diurnal skin depth were modified to simulate the formation or sublimation of interstitial ice. If a layer's daily maximum temperature was lower than the frost-point temperature of 198 K, its thermal inertia was slowly increased. The timescale for complete ice saturation was assumed to be 20 Mars years, which is long enough to cause negligible variations in soil thermal properties over diurnal and seasonal timescales. Layer thermal inertias were allowed to increase until they reached a maximum value of $1,256 \text{ J m}^{-2} \text{ s}^{-1/2} \text{ K}^{-1}$, which is $\sim 3/5$ the expected thermal inertia of pure solid water ice. If a layer's daily maximum temperature was higher than the frost-point temperature, then its thermal inertia was rapidly decreased to simulate ice sublimation. The timescale for complete sublimation was assumed to be 20 days. Experimentation showed that the results of these simulations were relatively insensitive to the timescales assumed, as long as the rates of ice condensation were assumed to be much longer than the rates of ice sublimation, which is likely to be the case because of the exponential dependence of equilibrium vapour pressure on temperature. f, g, Same as d and e, but using Ditteon's two-layer thermal and reflectance properties for low-thermal-inertia soil ($I = 79 \text{ J m}^{-2} \text{ s}^{-1/2} \text{ K}^{-1}$, $A = 0.344$), which provide a better fit to the IRTM brightness temperature observations²¹. f assumes a frost-point temperature of 198 K, whereas g assumes a frost-point temperature of 203 K.

to decrease average temperatures at depth while simultaneously insulating deeper layers from large-amplitude temperature variations. As long as the ice-free surface layer extends to a diurnal skin depth below the surface, the presence of high-thermal-inertia material below the surface has only second-order effects on diurnal variations in surface temperature. This means that, to first order, the properties of the ice-free surface soil layer control the daily average surface temperature and, at mid-latitudes, the annual average temperatures at depth. At latitudes where annual maximum subsurface temperatures are lower than the frost point, ice formation increases the thermal inertia and thermal diffusivity of subsurface soil layers. This causes the heat that is being conducted into these layers from above to be distributed deeply over a large thermal mass, decreasing the temperature variations that these layers experience, and promoting further ice formation. As the thermal inertias of the icy layers increase, they can migrate upward towards the surface by reducing the amplitudes of temperature variations in adjacent ice-free layers. As all of these effects tend to accelerate with increasing thermal inertia, martian ground-ice deposits would be expected to maximize their thermal inertias if water is available, and become fairly solid.

Actual ground-ice boundaries on Mars could deviate considerably from those calculated here because of variations and uncertainties in both the frost-point temperature and the thermal behaviour of the soil itself. The sensitivity of these boundaries can be demonstrated as follows. In low-thermal-inertia regions, measured infrared brightness temperatures at 6 p.m. local time can be more than 20 K colder than the temperatures predicted by simple homogeneous thermal models. Ditteon²¹ has fitted daily temperature variations observed over a complete diurnal cycle for a region in Tharsis to a r.m.s. accuracy of 3.14 K, using a thermal model that assumes a thin surface layer of bright low-thermal-inertia material overlying a deep layer of high-thermal-inertia material, which Ditteon attributed to the presence of duracrust or rock. When the same thermal model used in Fig. 2d and e is initialized using Ditteon's best-fit surface soil properties, but assuming that the high-thermal-inertia material below the surface is ground ice, the results in Fig. 2f are obtained. Increasing the assumed frost point from 198 K to 203 K gives the results shown in Fig. 2g, which would predict that near-surface ground ice is stable at the equator. Although Ditteon's parameters provide an improved fit to the IRTM observations, his explanation for the anomalously cold afternoon brightness temperatures is not unique, as they can be explained satisfactorily in many regions by the effects of diurnal variations in the rates of radiative and convective heat transfer between surface and atmosphere²². The extreme sensitivity of the results presented here suggests that a more complete treatment of all factors that influence subsurface temperatures and abundances of near-surface water vapour should be included in considerations of the stability of ground ice close to the equator.

Determining the present distribution of near-surface ground ice on Mars will be a prime objective for future spacecraft missions. Definitive evidence for its presence at low latitudes would enhance the prospects for human exploration and habitation. These results show that, all things being equal, low-thermal-inertia regions will be the most favourable locations for near-surface water-ice deposits. At present, low-latitude ground ice could be widespread within the Tharsis, Arabia and Elysium low-thermal-inertia regions. Over long timescales, the stability of ice in low-thermal-inertia regions is likely to be intimately linked with the martian climate, as the behaviour of both dust and water on Mars should be strongly affected by quasiperiodic variations in Mars's orbital and axial elements²³. In this respect, these regions may be similar to the layered deposits of dust and ice observed near the north and south poles²⁴. Although the nature of the martian environment during other climate epochs is difficult to predict, the possible stability of near-surface ground

ice in low-thermal-inertia regions on Mars today might help resolve a number of long-standing problems relating to the presence of low-latitude ice-related geomorphic features^{6-12,23,25-27}, and the possible involvement of ground ice in low-latitude sedimentary²⁸ and fluvial processes²⁹. □

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Forced vortex interaction and annihilation in an active medium

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THE formation of spatiotemporal patterns by coupling between diffusion processes and local, nonlinear reaction kinetics has been observed in diverse systems. In one of the most familiar, the autocatalytic oxidative bromination of malonic acid (the Belousov-Zhabotinsky (BZ) reaction¹), dynamical structures such as expanding target patterns and rotating spirals are observed. Most previous work on this system has been concerned with the autonomous dynamics of the travelling chemical waves, but more recently there has been increasing interest in the possibility of influencing the behaviour externally^{2,3}. Several studies have considered the effect of electric fields on spatial patterns in the BZ system⁴⁻⁸. We have investigated previously⁶ the influence of a homogeneous electric field on spiral waves. The spiral cores are of particular interest because they represent 'silent' centres in regions of pronounced dynamical activity⁹. Here we show that interactions between spiral cores can be induced and controlled by moving spirals towards each other using an applied field. Under carefully controlled conditions we have been able to induce mutual annihilation and transient coupling of spiral cores. Calculations using a simple model are able to reproduce the qualitative features of the experimental results.

The effect of electrical fields on spatial patterns in the BZ reaction was first studied by Feeney *et al.*⁴ and Ortoleva⁵. Ševčíková *et al.*^{7,8} found that in a small glass capillary, which

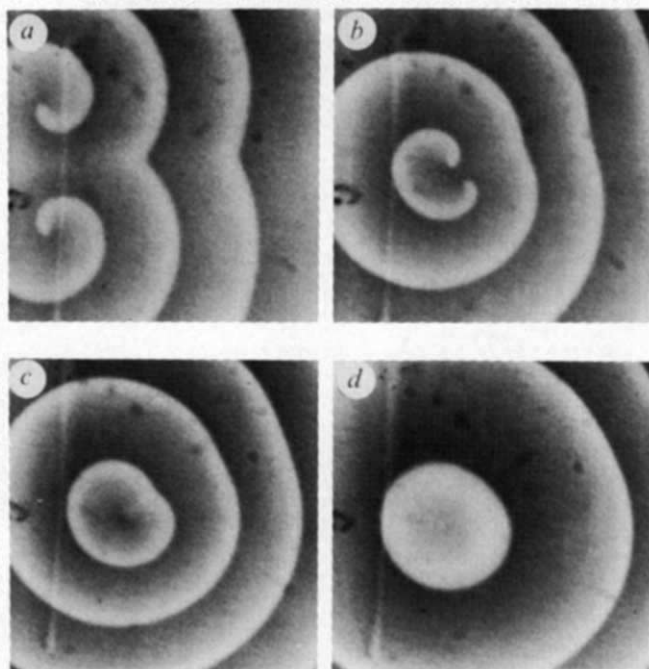


FIG. 1 Evolution of vortex annihilation. The electric field is oriented with the anode on the right and the cathode on the left. Opposite perpendicular motions of the vortex cores in the initial structure (a) reduce their separation. Instead of transient coupling (compare Fig. 2), annihilation occurs when the excitation travels through a small gap between the cores into a region of relatively low excitability (b, c). Bulk oscillations appear subsequently in the centre of the resulting pattern with low local frequency (d).

can be considered as a roughly one-dimensional system, applying an electric field can cause a chemical wave propagating towards the anode to accelerate. Changing the polarity may stop or split the wave, depending on the strength of the field. In the two-dimensional system introduced here, the external perturbation forces the spiral cores to drift towards the anode and also induces a perpendicular shift in a direction that depends on the chirality of the spirals. For a field orientation in which the cathode is on the left and the anode on the right, a clockwise-rotating spiral in the upper portion and a counterclockwise-rotating spiral in the lower portion of the system (seen from above) will be drawn together; when the polarity is reversed, they will be pulled apart.

The BZ reaction system was embedded in a 0.4% agar gel solution. The mixture contained 0.05 M NaBrO₃, 0.2 M H₂SO₄, 0.05 M CH₂(COOH)₂ and 6.25×10^{-4} M ferroin in a total volume of 5 ml, which was placed in a flat, rectangular dish (71.6 × 21.0 mm²), with a layer thickness of 3 mm. About 5 min after starting the reaction by adding the catalyst, a circular wave was triggered. A pair of spirals evolved when part of the wave front was stopped and disrupted by a vertically inserted removeable thin glass plate. The electric field was applied by means of two electrodes (distance ~70 mm) made of ion-free paper soaked with saturated K₂SO₄ solution⁶. A d.c. power source, used in

constant current conditions, supplied a weak electric field in the range 1.68 to 8.6 V cm⁻¹. Images were recorded with a CCD camera on a time-lapse video recorder and later stored into a video-frame buffer for further computational treatment.

We observed annihilation of spiral pairs when the electric field was of sufficient strength (>2 V cm⁻¹). The annihilation occurred when the symmetry axis of the spiral pair was nearly parallel to the field and, in addition, when the cores were not mutually screened through a difference in the phase angle of the two counter-rotating spirals. The four images in Fig. 1 show how the annihilation typically evolves. As the distance between the cores is reduced, the initial structure disappears. As can be visualized by superposing images⁹, annihilation takes place when the two individual cores still exist, indicating that the spiral cores do not simply coalesce. The conditions for annihilation depend on the (decreasing) distance between the spiral tips and the degree of excitability of the region enclosed by the innermost wave front. If the gap between the cores becomes sufficiently narrow ($\ll$ one wavelength), the remaining small chemical perturbation propagating into the area of low excitability disappears (Fig. 1c).

For the given medium, which is oscillatory with a rather long period, the final, almost circular wave (Fig. 1c) was followed by bulk oscillations occurring in the central region (Fig. 1d). Thus the annihilation process creates an autonomous pacemaker (region of wave initiation) for a target pattern. These target oscillations had previously been suppressed by the dynamic structures because of their higher frequency. In fact, for the high-frequency spiral structures the active medium behaves like an excitable system, because of the long period of the intrinsic oscillation. The centres of the resulting target patterns are, to our knowledge, the first reported example of pacemakers that clearly do not arise from inhomogeneities of the medium. We can specify their location by controlling the location of spiral annihilation.

Figure 2 shows the trajectories of a pair of coexisting spiral tips with different initial orientation, in an electric field of 5.6 V cm⁻¹. In this case the tips interact without annihilating. The three single frames in the background illustrate the appearance of the spirals before, during and after the interaction of the vortices. Under the influence of the field, the spiral cores immediately began to move along intersecting traces. When the distance was reduced to about one wavelength or less, visible interaction took place which prevented the cores continuing to drift in their original directions. Instead, the cores moved for a short distance along a common direction nearly parallel to the field. The cores attempt to avoid interacting by rearranging in such a way that the electric field will pull them apart. As shown in the final stage of Fig. 2, vortices of different topological charge are separated. The topological arrangement of the spirals necessary for this complex behaviour to occur—off-axis orientation of the spiral pair—was dictated by the procedure of spiral initiation, but a phase shift of the counter-rotating spirals might rise during the experiment as a result of slight inhomogeneities of the agar matrix.

We complemented the experiments by doing numerical simulations of the behaviour of spiral waves in an electric field. The intention was to extend a given two-variable reaction-diffusion model for an excitable medium¹⁰ by adding a gradient term

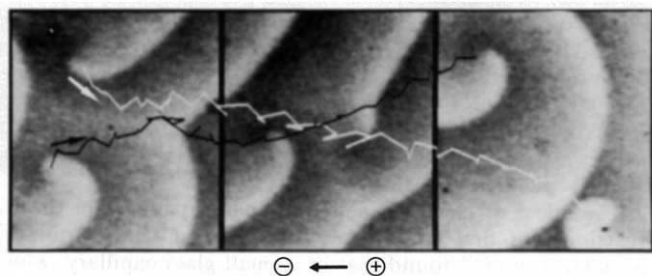


FIG. 2 Trajectories of two interacting vortices. The field is oriented so that the vortices, which are oriented off-axis and have different chirality, are forced to move on crossing traces. The interaction can be recognized as a deviation from the primary drift direction, with the new trajectories oriented nearly parallel to the field. Interaction ends when the vortex trajectories have crossed and start to diverge.

which describes the electric-field-induced current of a reactant. The model is based on the following equations:

$$\frac{\partial u}{\partial t} = f(u, v) + D\Delta u + ME \frac{\partial u}{\partial x}$$

$$\frac{\partial v}{\partial t} = g(u, v)$$

with $f(u, v) = \varepsilon^{-1}u(1-u)(u-(v+b)/a)$ and $g(u, v) = u-v$, where u denotes the fast excitation variable and v denotes the slow recovery variable. D is the diffusion coefficient of u , M the ion motility and E the electric field strength. $ME(\partial u/\partial x)$ is the additional flux term. The parameters a , b and ε control the dynamical properties (such as excitability) of the system¹⁰.

To compare the model with the BZ reaction, we take the autocatalytic species HBrO_2 as the excitation variable and the catalyst ferroin as the recovery variable. It might seem unreasonable to use an uncharged component to describe electrically induced flux, considering that more realistic models (such as the Oregonator) include the bromide ion, Br^- , which is most affected by the field. But in fact, because of the reaction kinetics, the dynamical behaviour of Br^- is closely related to that of HBrO_2 (ref. 1). The modified model used here allows a more general way of describing the effect of an external perturbation such as an applied electric field on a reaction-diffusion system.

The computer investigation agreed well with the experimental data. Figure 3 shows a graphical representation of an annihila-

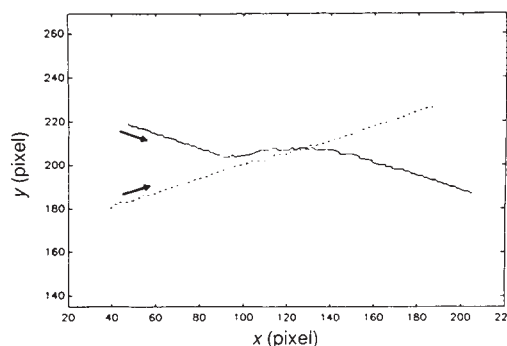


FIG. 4 Calculated traces of crossing vortices. The angle between the symmetry axis of the spiral pair and the field direction is 12° initially and -24° after the interaction. $ME/D=0.15$.

tion process. The trajectories of coexisting vortex tips are drawn in Fig. 4.

Electric fields can thus be used to influence and decompose dissipative structures in excitable systems by inducing drift of key ions, and could be a simple, controllable tool for investigating reaction-diffusion patterns. This could be useful, for similar studies of forced vortex interaction in other systems and could help to elucidate their specific dynamics^{11,12}. □

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Chemical tuning of electroluminescent copolymers to improve emission efficiencies and allow patterning

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ONE advantage of using conjugated polymers in semiconductor applications is that they can be processed using techniques well established for conventional polymers. We reported recently that poly(*p*-phenylenevinylene) could be used as the active layer in a light-emitting diode¹, producing yellow/green emission. We have now found that related copolymers, comprising a combination of different arylene units, can be chemically tuned to provide a range of materials with considerably improved properties for this and other applications. By incorporating two different leaving groups into a precursor copolymer, we can selectively eliminate one of these, to give a conjugated/non-conjugated copolymer, or both, to give a fully conjugated copolymer. This allows us to induce local

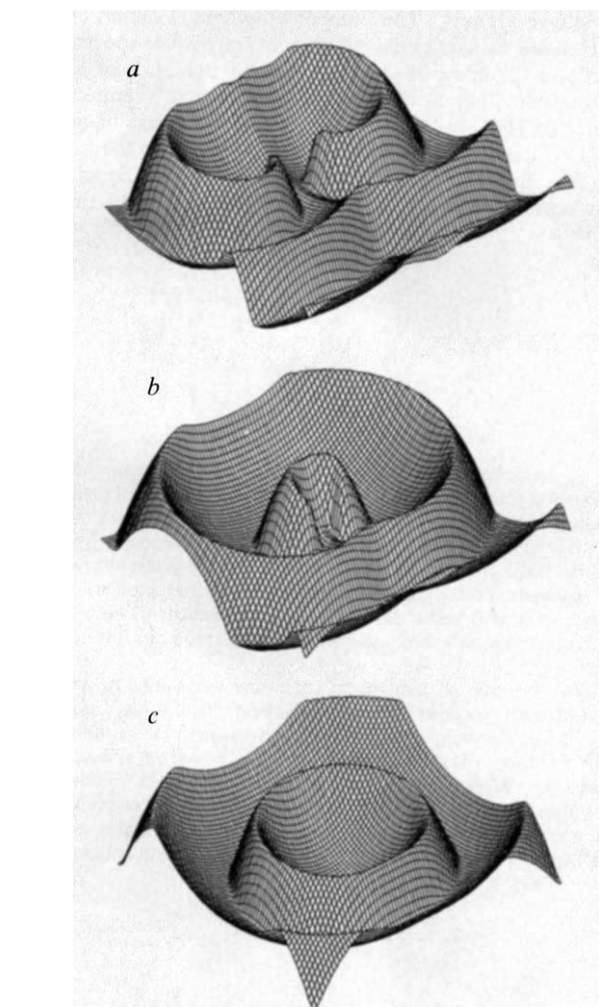


FIG. 3 Three-dimensional representation of the slow recovery variable, v , in a numerical simulation of annihilating vortices with $ME/D=0.3$. *a*, Initial structure. *b*, Region enclosed by the vortex pair is small and has low excitability. *c*, Chemical perturbation disappears in the centre of an expanding circular wave.

variations in the π - π^* electronic energy gap at both the molecular and supramolecular level. Variations at the molecular level can act to trap excitons, hindering their migration to quenching sites, and we find that these materials give strongly enhanced quantum yields for electroluminescence (by a factor of up to 30). They also allow control of the colour of emission. Variations at the supramolecular level, by patterning the films to control the progress of conversion, allow the production of structures suitable for multicolour displays. The ability to pattern the film also allows for fabrication of optical waveguides, as regions with different energy gaps have different refractive indices.

Much attention has recently been devoted to the development of efficient routes to conjugated polymers²⁻⁹. Because most organic materials with a high level of conjugation show poor solubility in common solvents, approaches to conjugated polymers have involved the preparation of a processible precursor polymer, the attachment of groups that increase solubility^{8,10}, or a combination of both.

In our initial work¹ on the light-emitting properties of poly(*p*-phenylenevinylene) (PPV) we observed that the efficiencies (up to 0.01%; photons emitted per electron injected at room temperature) depended on the combination of electrode materials used. We used aluminium with a surface oxide coating or indium/tin oxide as the positive, hole-injecting contact, and aluminium as the negative, electron-injecting electrode. We found that a segmented PPV analogue emitted blue-green light (508 nm) with a twofold improvement in efficiency¹¹. Considerably higher efficiencies are obtained with metals of lower work function as the negative electrode: Braun and Heeger¹², working with a soluble dialkoxy derivative of PPV, showed that efficiencies could be raised to 1% through the use of calcium, whose lower work function allows much easier injection of electrons. We find that efficiencies can be raised to above 1%, using some of the copolymers discussed below. This illustrates the importance of controlling the injection electrodes, and we see scope

for further improvements by using hole and electron transport layers. Here, however, we are concerned with the optimization of the emissive layer formed from the conjugated polymer.

Both photoluminescence and electroluminescence originate from the radiative recombination of exciton states, in the first case formed by photoexcitation, and in the second by combination of oppositely charged polarons (radical ions) generated by injection of electrons and holes¹³. The quantum yield for photoluminescence in small conjugated molecules can be very high, but the yield in PPV is reduced as the extent of π -electron conjugation is increased¹⁴. Increased mobility of excitons in the delocalized π -electron system will allow more rapid motion to quenching centres; among the latter, self-localized charged excitations have been experimentally identified as important^{15,16}. We set out, therefore, to introduce local variations in the potential that could act as exciton traps and reduce motion to quenching sites, by synthesizing copolymers that have regions of different π - π^* energy within a single chain.

The essence of the synthetic route was to produce a precursor polymer polyelectrolyte with two different leaving groups on the main chain. We illustrate this here for the monomers (I) and (II), copolymerized to form polymer (III), which was then converted to polymers (IV) or (V) by heat treatment under different conditions. Formulae and preparation details are given in Fig. 1. We chose these two monomers because their conjugated homopolymers show substantially different bandgaps, 2.5 eV (496 nm) for PPV and 2.1 eV (590 nm) for poly(2,5-dimethoxy-*p*-phenylenevinylene)¹⁷. The films of polymers (IV) and (V) were characterized by infrared and ultra-violet/visible spectroscopy. The infrared spectra of (IV) showed the presence of methoxy leaving groups, which disappeared on thermal treatment in the presence of HCl. In the optical absorption spectra of the polymers (IV), the energy of the band edge shifted to the blue with increasing numbers of dimethoxy-*p*-phenylene units. These results confirmed that the polymer (IV) had been randomly

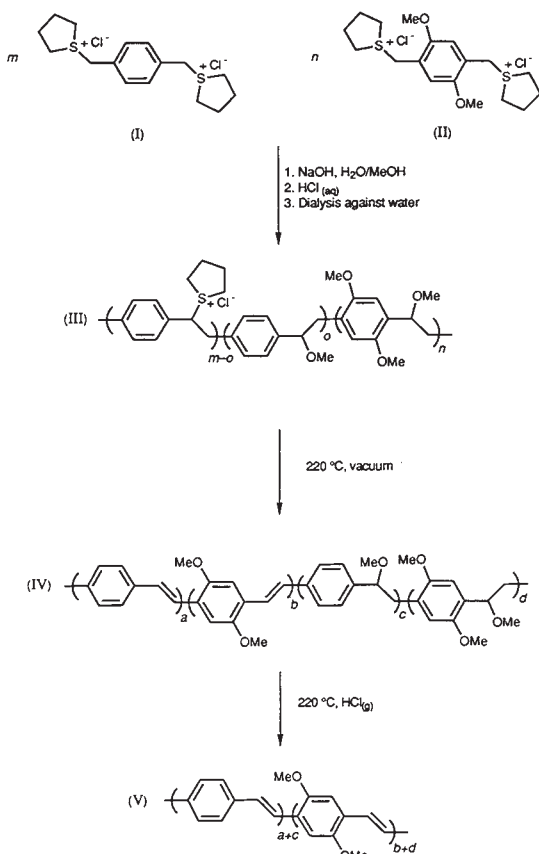


FIG. 1 Synthetic pathway. The symbols *m* and *n* represent the molar equivalents of the monomeric salts (I) and (II) in the reaction. A small number (*o*) of benzylic positions adjacent to the phenylene rings in (III) are substituted by methoxy groups. Thermal elimination of all the sulphonium groups and a few methoxy groups from (III) produces the polymer (IV), which has an arrangement of conjugated units randomly interrupted by saturated units (where *a*, *b*, *c* and *d* stand for the total number of each structural type). Further acid-promoted conversion of (IV) produces the conjugated polymer (V).

METHODS. The ratio of units in the copolymer was varied by altering the amount of each monomer unit in the reaction mixture. We copolymerized the two *bis*(sulphonium) salts in a water/methanol mixture with aqueous sodium hydroxide. After termination, polymers (III) were purified by dialysis against water before isolation and dissolution in methanol. Thermal conversion of films of polymers (III) (220 °C *in vacuo*; 2 h) produced homogeneous, dense and uniform films of the polymers (IV) (typical thickness 100 nm). The polymers (V) were formed from (III) by heat treatment under acidic conditions (220 °C, HCl/Ar, 22 h).

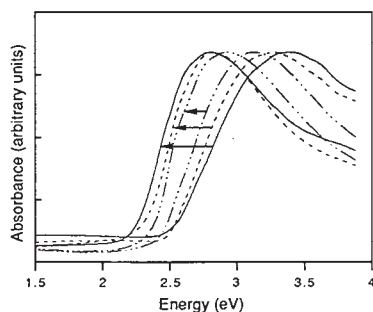


FIG. 2 Absorption spectra of copolymers prepared from ratios of (I) to (II) 9:1 (---), 4:1 (---), 7:3 (—) from thermal treatment and acid and thermal treatment. The shift is indicated in the direction of the arrows for each case.

segmented into discrete conjugated units, because the sulphonium groups on the benzylic carbons attached to the 2,5-dimethoxy-*p*-phenylene units are readily displaced by methanol. Those attached to the benzylic carbons of the *p*-phenylene units are not. During the thermal conversion, the sulphonium groups are readily lost, whereas the methoxy groups resist elimination. They require both heat and acid. Consequently thermal and acidic treatment of the polymer (III) yields the polymer (V). Elimination of both the sulphonium and methoxy leaving groups is substantially complete, with the result that the energy gap shifts to the red (Fig. 2).

We thus have the choice of conjugated copolymers (V) or conjugated/nonconjugated copolymers (IV). We have looked at the properties of the conjugated/nonconjugated copolymers in electroluminescent diode structures, and find that these offer better device efficiency than PPV. The most striking results are obtained for devices with aluminium/aluminium oxide and aluminium contacts. The device efficiency depends strongly on the monomer feed ratio, increasing from 0.01% (PPV in the same device configuration) by a factor of up to 30 (to 0.3%) as the monomer feed ratio (I):(II) was reduced to 9:1; copolymers arising from lower feed ratios (I):(II) showed an efficiency that decreased consistent with the lower photoluminescence yield from poly(2,5-dimethoxy-*p*-phenylene vinylene)¹⁸.

Conversion of (III) to (V) can be achieved lithographically, by control of the presence of acid through patterning, and thus copolymer samples with supramolecular variations in π - π^* energy gap can also be prepared. One convenient method is to make use of the hydrogen chloride, a by-product of the thermolysis of the sulphonium groups of polymer (III), to catalyse the removal of the methoxy leaving groups. This merely requires that the acid generated in this way is trapped in the film. We achieved this by masking a thin film of the precursor polymer

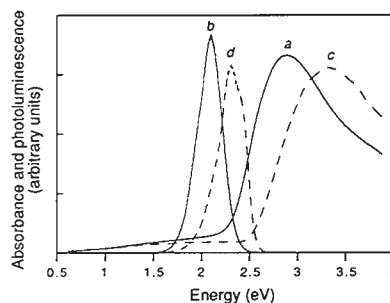


FIG. 4 Absorption (a) and photoluminescence (b) spectra of a capped sample, and absorption (c) and photoluminescence spectra (d) of an uncapped sample of copolymer made from a 7:3 mixture of (I) and (II).

(III) with strips of evaporated aluminium or another suitable metal (Fig. 3). After thermal treatment and removal of the aluminium mask, the films showed a striking difference in colour (orange where previously coated and yellow elsewhere) and in their photoluminescence spectra (Fig. 4). Such a process is readily amenable to lithographic techniques, with resolution limited only by acid diffusion around the edges of the mask.

We compared the effective refractive indices of the TE and TM optical waveguide modes in each of two closely neighbouring regions on either side of the boundary at a wavelength of 632.8 nm, and we estimated the difference in the polarization-dependent refractive index n_{TE} and n_{TM} in the two regions. We found a striking difference in n_{TE} (electric field parallel to surface) linked to the change in absorption spectra in the two different regions. For example, a copolymer prepared from a 4:1 feed ratio of (I) to (II) treated in the above fashion had $n_{TE}=1.81$ in the uncapped region (polymer absorption maximum at 376 nm (3.30 eV)) and $n_{TE}=1.96$ in the capped region (polymer absorption maximum at 427 nm (2.90 eV)).

The efficiency of the output of electroluminescent devices that incorporate copolymers can be raised to levels comparable to those of inorganic devices operating in the yellow-green part of the spectrum. Furthermore, the colour of the electroluminescent output can be varied by controlling the chemistry behind the copolymerization and the degree of conversion to the conjugated form. Emission over the spectral range from green-blue¹¹ to orange-red has already been demonstrated using different copolymers. Supramolecular control of refractive index through control of the π - π^* energy gap also offers scope for guided-wave devices. □

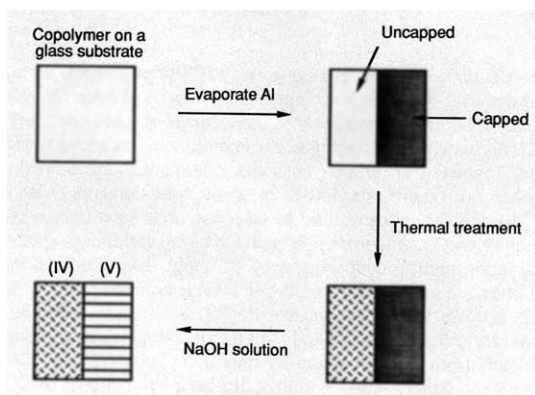


FIG. 3 Schematic diagram showing the process for forming a pattern of polymers (IV) and (V) from polymer (III).

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Interhemispheric asymmetry in OH abundance inferred from measurements of atmospheric ^{14}CO

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THE hydroxyl radical, OH, is the chief oxidizing agent in the atmosphere, and is responsible for removing many natural and anthropogenic trace gases¹. At present, OH cannot be measured directly with sufficient accuracy over the spatial and temporal scales needed for global models of atmospheric chemistry². Consequently, estimates of atmospheric OH abundance rely on a combination of models incorporating OH chemistry and observations of trace gases sensitive to OH. ^{14}CO is an important diagnostic of OH abundance³⁻⁶. It is produced in the atmosphere mainly by the immediate oxidation of ^{14}C produced by cosmic radiation, and it is subsequently removed more slowly through oxidation to $^{14}\text{CO}_2$ by hydroxyl radicals⁷. The mean lifetime of ^{14}CO in clean air during summer in the middle and low latitudes is about one month, which makes ^{14}CO a more sensitive indicator of OH than the longer-lived trace gases commonly used. Until now, only a few Northern Hemisphere ^{14}CO determinations have been published^{3,8}. Using accelerator mass spectrometry we present here an extensive set of ^{14}CO data in New Zealand and several new Northern Hemisphere results. We find that Southern Hemisphere ^{14}CO concentrations are $\sim 40\%$ lower than at comparable latitudes in the Northern Hemisphere. Such a large difference is surprising because the dominant sources and sinks are believed to be similar in both hemispheres. Although there are several complicating factors, from our results we suggest that OH abundances may be significantly higher in the Southern Hemisphere than in the Northern Hemisphere, in contrast to predictions using current photochemical models.

We extracted CO *in situ* from 1.5-m³ air samples following the method of Stevens and Krout⁹. In essence, after removal of at least 99.9999% of atmospheric CO₂, the CO content is oxidized over I₂O₅ to CO₂, which is subsequently trapped cryogenically. This quantitative extraction procedure provided 60–225 μl of CO-derived CO₂, depending on the wind direction during sampling and the time of year. After determination of the ratios $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$ by mass spectrometry, we diluted the small samples with ^{14}C -free CO₂ and then graphitized them¹⁰ for accelerator mass spectrometry (AMS) ^{14}C analysis¹¹. The systematic maximum uncertainty in determination of ^{14}CO abundance is less than 6% as derived from the combined uncertainties in the CO concentration itself, the dilution factor and the AMS ^{14}C measurement. A second conversion/extraction system has been used for processing air collected in cylinders. By processing duplicate air samples, we established that the standard deviation of a ^{14}CO measurement was 3%. The stable isotope results, which are of relevance to understanding the CO cycle in the SH, and details of analytical procedures will be published elsewhere¹².

Samples were collected at our clean-air station Baring Head (41°S) between June 1989 and February 1991. The specific ^{14}C activity of atmospheric CO in clean surface air is very high. A CO sample collected at Baring Head on 21 July 1989 contained 841 pM (% modern carbon¹³), whereas Southern Hemisphere atmospheric CO₂ contains 117 pM. The corresponding CO con-

centration of 58.0 p.p.b. (parts per 10⁹) leads to a ^{14}CO abundance of 15.1 molecules per cm³ of air (STP). ^{14}CO and CO values for Baring Head are shown in Fig. 1.

Concentrations of ^{14}CO measured at Baring Head are almost a factor of 2 lower than those determined by Volz *et al.*³ at 51°N during the years 1976 to 1978 (see upper panel of Fig. 2), namely 6.3 ± 0.3 compared with 11.5 ± 1.0 molecules cm⁻³ in summer, and 12.9 ± 1 compared with 25 ± 2 in winter. This large ^{14}CO difference between Northern and Southern Hemispheres is of special interest in view of the potential asymmetry in OH levels between the hemispheres. Higher Northern Hemisphere concentrations of nonmethane hydrocarbons and CO have been considered to lead to much lower OH concentration¹⁴, a view which has been changed in favour of higher OH concentrations in the Northern Hemisphere largely because of mitigating effect from high NO_x (NO + NO₂) emissions¹⁵. Distributions of OH obtained from current models of OH chemistry vary considerably but generally have interhemispheric asymmetry of less than 20%.

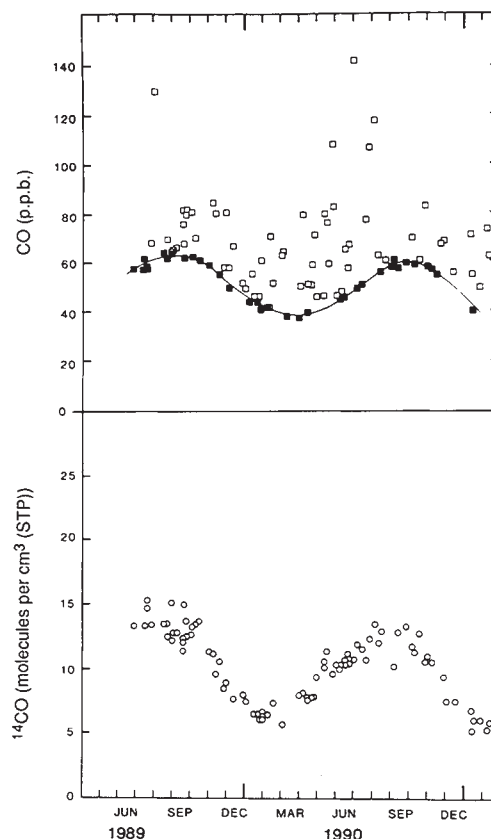


FIG. 1 The CO concentration at Baring Head (□) shows large fluctuations depending on wind direction and speed. Clean air conditions (■) with low CO occur during southerly winds only³⁵. Our clean air, absolute concentration data for CO agree within 4% with gas chromatographic measurements from Cape Grim, Tasmania (P. Fraser, personal communication). Sampling and analysis have not been restricted to clean air conditions, in order to link ^{14}C , ^{13}C , ^{18}O and CO concentration to air-mass origin and source isotopic compositions. The CO seasonal cycle is driven by OH variations, which affect both destruction and production, and by CO input from biomass burning. ^{14}CO (○) shows a larger seasonal cycle, almost entirely due to seasonal OH variations. Note that the variability of ^{14}CO is not large compared with CO. The reason is that high CO values are due to sampling air contaminated with CO mainly from fossil fuel combustion, which is devoid of ^{14}CO . Thus ^{14}CO is a tracer largely independent of the local air content of CO. High ^{14}CO values, particularly in winter, are associated with gale-force southerly winds. Air back-trajectory analysis shows that air masses from as far as 70°S can reach Baring Head within three days.

The large difference between the recent Southern Hemisphere and older Northern Hemisphere data sets may partly reflect changes in OH over the 12-yr period. Different techniques have also been used, and the earlier results have a larger uncertainty associated with them. An important consideration is that ^{14}C production varies with the degree of solar activity. The 11-yr solar cycle has two effects on the ^{14}C production rate^{16,17}. Less ^{14}C is produced by galactic cosmic rays during a solar maximum, as occurred around 1989–90, whereas additional ^{14}C may be produced by protons from solar flares which are more frequent at that time. By using O'Brien's relationship¹⁶ between sunspot number R and ^{14}C production Q ($\text{atom cm}^{-2} \text{s}^{-1}$), $Q = 1.937 - 0.00242R$, we calculate that galactic cosmic ray ^{14}C production was $\sim 12\%$ lower during the period considered than in the earlier period of Northern Hemisphere measurements³. On the other hand, by calculating the ^{14}C production induced by solar protons¹⁷, we estimate that the high-energy solar events of the last half of 1989 (refs 18, 19) have produced additional ^{14}C equivalent to 12–27% of a year's production of ^{14}C . But the distribution in the atmosphere of this additional ^{14}C differs from that of ^{14}C produced by galactic cosmic rays, and production occurred during a few very short events. Altogether the above factors make detailed comparison of the 1989–90 and 1976–78 data difficult.

We have therefore collected five air samples at a location in the Northern Hemisphere, the Schauinsland monitoring station in Germany (48°N , 8°E , 1,200 m above sea level). As can be seen from Fig. 2, these new measurements imply somewhat reduced ^{14}CO concentrations, in particular in winter. Differences of this magnitude can be expected as a result of changes in solar activity and the other uncertainties mentioned. Basically, our recent measurements confirm a large ^{14}CO difference between Northern and Southern Hemispheres, 11.2 ± 1.0 compared with 6.3 ± 0.3 molecules cm^{-3} in summer and 20.1 ± 1.6 compared with 12.9 ± 1.0 molecules cm^{-3} in winter. Here we consider only the recent measurements which represent locations of comparable, but not identical latitudes in both hemispheres.

Because ^{14}C production is greater towards the poles and, conversely, ^{14}CO destruction by OH is greater towards the Equator, a latitudinal gradient is expected. Therefore we first estimate the effect on ^{14}CO caused by the modest difference in latitudes between the two sampling locations, 48°N and 41°S . The latitudinal variation of ^{14}CO was estimated by Volz *et al.*³ using the Atomic Energy Research Establishment two-dimensional model, and a ^{14}CO gradient of 2–3% per degree of latitude was derived. Results for a few samples collected by Volz in the Atlantic and Mediterranean indicate the presence of a weaker ^{14}CO latitudinal gradient. In the Southern Hemisphere, gradients are much smaller. Three-day back-trajectories for air sampled at Baring Head show variations in air-mass origin of more than 30° latitude, ranging as far south as 70°S . The corresponding variations in ^{14}CO are ~ 1.5 molecule cm^{-3} , implying gradients of only 0.6% per degree of latitude or less. This is confirmed by analysis of aircraft samples taken between New Zealand and Antarctica, and in Antarctica itself (J. E. Mak, unpublished results). Thus although the latitude effect is difficult to quantify, the 7° difference between the Northern and Southern Hemisphere locations is unlikely to explain more than $\sim 10\%$ of the observed ^{14}CO difference.

The most important factors that can cause higher ^{14}CO levels in the NH are (1) greater cross-tropopause transport of ^{14}CO from the stratosphere, where production rates are high; (2) higher concentrations of natural nonmethane hydrocarbons which on oxidation give additional ^{14}CO and (3) lower OH concentrations. We have used a two-dimensional box model of the atmosphere to construct self-consistent budgets for ^{14}CO over broad latitude bands. Although this does not show details of latitudinal gradients, it does allow careful calibration of the model with respect to the first two factors. The model is based on that of Cunlold *et al.*²⁰. The troposphere is represented by

a total of eight boxes defined by 90° , 30° and 0° latitude in each hemisphere, pressure levels of 1,000 and 500 mbar, and a tropopause at 120 mbar (0° to 30°) and 230 mbar (30° to 90°). We introduced separate stratospheric boxes for each hemisphere and a seasonally varying stratosphere–troposphere exchange determined by requiring the model to simulate $^{14}\text{CO}_2$ surface values in the wake of nuclear weapons tests of the mid-1960s (refs 21, 22). This is a pertinent test of cross-tropopause transport of ^{14}CO , as by 1965 the excess $^{14}\text{CO}_2$ was peaked at the altitudes of maximum ^{14}CO production²³. Exchange parameters were based on Holton's estimates²⁴ of large-scale downward fluxes in the lower stratosphere, with additional exchange in spring and summer to incorporate the effect of smaller-scale cross-tropopause transport components. The resulting cross-tropopause transport is twice as large in the Northern as in the Southern Hemisphere, in agreement with independent estimates based on global ozone budgets²⁵.

We used Lingenfelter's ^{14}C production rate distribution²⁶ scaled down to a global average of 1.8 ^{14}C atoms $\text{cm}^{-2} \text{s}^{-2}$ (ref. 14), and a 90% yield for ^{14}CO formation⁸. Recycled ^{14}CO from sources of CO containing ^{14}C has been included by constructing a total CO budget with sources due to methane oxidation²⁷, nonmethane hydrocarbon oxidation²⁸, biomass burning²⁹ and fossil carbon sources. The ^{14}C content of the nonfossil carbon sources is taken as 120 pM. The OH sink is derived from the seasonal and latitudinal OH distribution of Spivakovsky *et al.*³⁰ scaled by 0.9 to match the observed methyl chloroform lifetime³⁰. In the tropospheric boxes used here, this gives annual average OH densities of 7.8×10^5 and 7.6×10^5 radicals cm^{-3} for the Northern and Southern Hemispheres respectively. The CO concentrations obtained in the two hemispheres are consistent with observations, and thus the recycled ^{14}CO source has roughly the right magnitude and latitudinal variation.

The model-derived ^{14}CO concentrations (Fig. 2) in the 30° to 90° lower-troposphere box for the Southern Hemisphere are $\sim 10\%$ higher than ^{14}CO concentrations observed in New Zea-

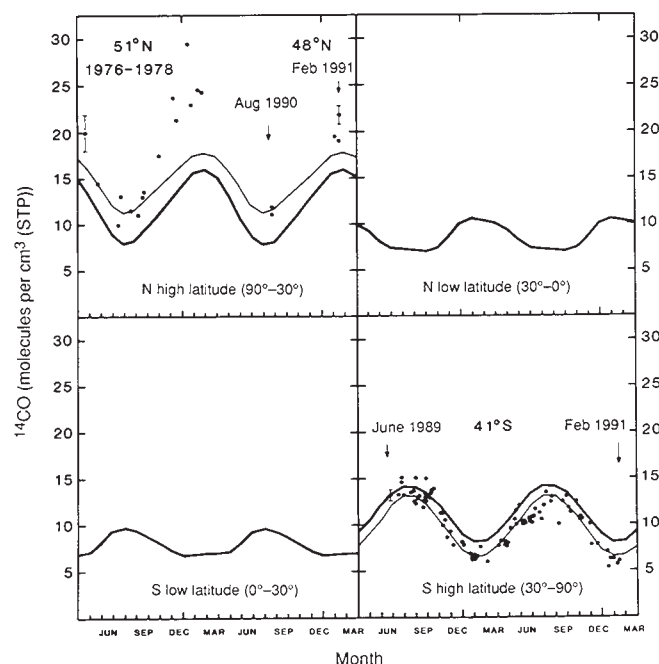


FIG. 2 Model results for the annual cycle of ^{14}CO in the four lower-tropospheric boxes, together with the present data and that of Volz *et al.*³, expressed as molecules per cm^3 at STP. The bold line gives the reference case with OH taken from Spivakovsky *et al.*³⁰, multiplied by 0.9. The thin line is for the case in which OH was decreased in the Northern Hemisphere and increased in the Southern Hemisphere, as discussed in the text.

land, whereas the corresponding model values for the Northern Hemisphere are 20–30% lower than those observed in Germany. Thus, although the model allows for higher cross-tropopause transport and recycled ^{14}CO levels in the North, it fails to explain the extent of the observed asymmetry between the hemispheres. Applying the estimated maximum correction due to the latitudinal difference between sampling locations still leaves a discrepancy of 20–30% unaccounted for.

Without more data on the spatial distribution of ^{14}CO and the help of a three-dimensional transport model, we cannot completely rule out additional factors that could have contributed to the higher observed ^{14}CO levels in the Northern Hemisphere. One is ^{14}CO release from nuclear power plants, which is negligible on a global and regional scale³, but may have a local effect in Germany. The other is that recycled ^{14}CO from biogenic sources may not be as evenly distributed as implicitly assumed in the model. Thus locally higher CO concentrations at the German sites may contain more recycled ^{14}CO . But most samples from Schauinsland station were collected when the collection site was separated from the boundary layer by an inversion underneath. Furthermore, a sample from the northern Atlantic at 43° (ref. 3) did not give a value lower than expected on the basis of the samples from Germany. It is therefore improbable that the recent Northern Hemisphere data are significantly affected by local sources. Although one could speculate that an unrecognised Southern Hemisphere sink for CO exists, we believe consideration should be given to explaining the observed interhemispheric ^{14}CO asymmetry in terms of OH distribution.

To evaluate the sensitivity of ^{14}CO to OH, we consider the effect of scaling the OH field by factors of 0.6, 0.8, 1.0 and 1.2 in the 90–30° N, 30–0° N, 0–30° S and 30–90° S bands. This change leaves the total global OH inventory very close to that used in the reference case in which the OH field was scaled everywhere by 0.9. The outcome, shown in Fig. 2, indicates that this is the scale of OH asymmetry implied by our analysis, although the matching of the Northern Hemisphere ^{14}CO seasonal cycle is not complete.

There are several reasons why global-scale models of OH chemistry may not predict interhemispheric asymmetry correctly. For instance, the significant effect of higher concentrations of nonmethane hydrocarbons in the Northern Hemisphere is difficult to incorporate and is sometimes ignored. It is difficult to pinpoint a difference between the hemispheres that could lead to substantially more OH in the Southern than in the Northern Hemisphere. Of topical concern is the possible role of stratospheric ozone depletion in the Antarctic, which enhances ultraviolet fluxes, possibly leading to higher OH production. Recently, Schnell *et al.*³¹ reported that tropospheric chemistry in Antarctica has changed, probably because of stratospheric ozone depletion. For the Northern Hemisphere, Liu *et al.*³² report that because of abundant anthropogenic aerosol, the ultraviolet flux at ground level may have decreased. This effect could have a role over large areas and affect OH distribution. Two more, recently reported, differences linked to OH chemistry are worth mentioning. Kelly *et al.*³³ report a large asymmetry in upper-troposphere H_2O abundance which would tend to reduce OH production rates in the Southern Hemisphere, where they found less water vapour. This difference is only documented, however, for winter, when ultraviolet irradiance and hence OH production are low. Finally, Jacob and Klockow³⁴ found during an Atlantic cruise that H_2O_2 concentrations are considerably higher in the Southern Hemisphere. □

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Material flux and porosity changes during sediment diagenesis

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UNDERSTANDING the changes in porosity that occur during burial of sediments is of importance both in modelling fluid flow in sedimentary basins and for prediction of petroleum reservoir quality. When deposited, up to 50% of the volume of sands is intergranular pore space. Porosity generally decreases with depth because of compaction and precipitation of diagenetic minerals, but some sandstones retain an anomalously high porosity. Whether this is due to dissolution and removal of material or to its local redistribution is not clear. We originally intended to quantify losses of major chemical components during burial of a complete sedimentary unit. We have investigated ten reservoir sandstones, of Permian to Tertiary age, from oilfields worldwide. These show consistent results, and here we present detailed data from four of them. We found that the silica content of these sandstones actually increased, by 220 to 350 kg m⁻³, following compaction. No statistically significant changes were observed for aluminium, potassium or sodium. The flux of silica cannot be modelled satisfactorily using currently accepted values of permeability, silica solubility and flow rates, posing a challenge for existing models of fluid flow and ore emplacement.

Empirically derived curves have been used to describe the decrease of sandstone porosity with depth¹. Anomalously high porosities may be caused by dissolution of pre-existing grains and cement (secondary porosity), or inhibition of cement growth by clay coatings on grains. Schmidt and McDonald² attributed enhanced porosity in deeply buried reservoir sands to leaching of feldspar grains and carbonate cement by CO₂-rich, aggressive solutions, but did not establish whether porosity was being

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TABLE 1 Worked example of imports and exports to or from the Garn Formation

	SiO ₂ (Wt%)	Al ₂ O ₃ (wt%)	Na ₂ O (wt%)	K ₂ O (wt%)	Porosity (%)
initial	62.86 ± 3.86	3.82 ± 0.81	0.32 ± 0.07	1.98 ± 0.55	31.0 ± 4.00
final	76.85 ± 3.99	2.98 ± 1.17	0.13 ± 0.10	1.13 ± 0.40	18.9 ± 3.50
import (+) or export (-) (%)	18.9 ± 8.4	-0.22 ± 1.18	-0.12 ± 0.10	-0.45 ± 0.54	

The figures for import/export for SiO₂ and Na₂O are calculated net changes, whereas the values for Al₂O₃ and K₂O give upper limits for net change. We analysed 55 samples. All errors are quoted at 95% confidence limits. The change in abundance of an oxide from deposition to the present day was calculated from the equation $\Delta X_i = (X_i(1 - f_i)) - (X_i(1 - f_i))$, where ΔX_i is the true change in oxide abundance (% total rock plus void volume), X_i is the final (present-day) oxide abundance (% of rock only), X_i is the initial oxide abundance (% of rock only), f_i is the present fractional porosity and f_i is the fractional porosity just before cementation.

redistributed rather than created by removal of material from the reservoir unit. In contrast, Bjørlykke and co-workers^{3,4} considered that the reservoir is an isochemical system in which chemical components are redistributed. The generic problem is to understand over what distance material is being redistributed, and thus whether the system can be considered to be isochemical on the scale of hand-specimen, bed, formation or an even larger dimension.

Changes in a rock sequence are quantified by comparison with the sediment as it was before the diagenetic changes occurred. In work on clay-mineral transformations in mudrock systems^{5,6}, samples from a large vertical section were compared with the reasonable assumption that rocks of apparently the same depositional facies had the same initial mineralogy. This assumption is not rigorous, as it is not possible to assign uncertainties to variations in original chemical composition and thus constrain the significance of calculated differences after diagenetic change. The alternative is to use a lateral rather than vertical equivalent⁷; but here changes in lithofacies often complicate the issue.

Some concretions, which form soon after deposition of a sediment, preserve both the depositional fabric and the composition of the sand grains. Others preserve neither of these; the concretionary cement, in part, replaces the host sediment. We took care to obtain the data reported here from concretions in which petrographic analysis had shown the original sand grain to be in pristine condition and unaffected by entombment in carbonate cement. The sandstones studied here were from the following oilfields: North Sea Central Graben (Permian), North Sea Sole Pit Basin (Triassic), Northwestern Australian Shelf (Triassic), North Sea Viking Graben (Jurassic), Norwegian Hammerfest Basin (Jurassic), Western Canada Basin (Cretaceous), United States Gulf Coast (Tertiary) and Central Sumatra (Tertiary). Evidence of early growth comes from textural observations and stable-isotope data⁸⁻¹⁰. Similar criteria have been used to understand concretionary growth in mudstones¹¹⁻¹³.

We devised a simple method to allow full quantification of the import/export patterns of elements that occur mainly in the silicate fraction of a sandstone (Si, Al, K, Na). We calculate changes in chemical composition for a unit volume of rock, correcting for compaction of that volume during burial. Four pieces of data are required for this calculation: the present chemical composition of the sandstone; the initial (depositional) chemical composition of the sandstone; the present porosity of the sandstone; and the porosity of the sandstone immediately before cementation. The present porosity and chemistry of the sandstone can be measured directly^{14,15}. The initial chemistry of the silicate fraction can be measured by analysing the sediment trapped in the early diagenetic carbonate concretions.

The effects of compaction, that is the difference between present porosity and porosity just before cementation, may be calculated in two ways. The first uses the fact that titanium mobility is limited during diagenesis¹⁶. In a uniform system the ratio of TiO₂ concentration in the host sandstone (corrected to exclude carbonate) is directly proportional to the porosity loss due to compaction. The second method is to use an empirical porosity/depth equation^{1,17}. Depth cannot be determined

directly but may be related to time or temperature of diagenetic mineral growth using burial and thermal history models. Dates and/or temperatures of silicate precipitation are determined from radiogenic isotope dating or fluid inclusion analysis, respectively. Where results can be checked independently, different methods are consistent.

The method above assumes that the initial chemistry of the sandstone is preserved in the concretions, and that the detrital sediment in the concretions was, at deposition, the same as the rest of the sediment. Only the earliest diagenetic concretions were chosen for analysis: those containing ~40% calcite cement (that is, preserved depositional porosity¹⁸) and a carbon/oxygen isotope signature typical of an origin at shallow depth by bacterial processes^{9,10}.

The preserved sediment in the concretions is not unusual and the concretionary horizon is not atypical. Initial studies were done on the most homogeneous, quartzose sandstones available, the Upper Jurassic Brae Formation sandstones of the UKCS Miller Field. We have compared the chemistry of sand inside and outside nodular concretions in sandstones that have no other cements (shallowly buried Garn and Rogn sandstones of the Middle/Upper Jurassic Haltenbanken NOCS). The chemical compositions are not significantly different (we analysed Si, Al, Na and K in the carbonate-free fractions of four concretions, and 12 samples of sandstone). Published data¹⁹ indicate that concretions are randomly distributed in concretion-rich horizons and the chemical compositions of sands adjacent to concretions and their vertical equivalents are not significantly different.

We present the calculated elemental import and export in detail for four sandstone types: quartzose from the Upper Jurassic Brae Formation, North Sea (two segments at 4.2 and 4.7 km); sub-arkosic from the Upper Jurassic Ula Formation, North Sea; sub-arkosic/sub-lithic Middle Jurassic Garn and Rogn Formations, offshore from mid-Norway.

Each of these formations is relatively homogeneous within the two main lithofacies, sandstone and mudstone. They are Jurassic sandstones and cover a depth range of 1.5 km to 4.7 km and a range of present-day temperatures from 40 to 150 °C. About 50 samples were selected from about five wells in each of the sandstones. The depths of the samples were selected to be statistically random. Successive samples were selected until the porosity and permeability (poroperm) distributions for the selected set could not be distinguished from those of the whole

TABLE 2 Summarized imports and exports from reservoir sandstones

	SiO ₂	Al ₂ O ₃	Na ₂ O	K ₂ O
Brae, 4.2 km	+297 ± 149	+4.2 ± 39	+0.3 ± 3.8	+0.7 ± 11
Brae, 4.7 km	+350 ± 157	+7.1 ± 38	+0.8 ± 4.1	+0.5 ± 7.3
Ula	+174 ± 198	+6.7 ± 29	+0.5 ± 11	+3.5 ± 14
Garn	+353 ± 156	-4.1 ± 22	-2.2 ± 1.9	-8.4 ± 9.9

These estimates are based on chemical analyses of early-cemented and equivalent uncemented rocks. All values are given in kg m⁻³ and errors are 95% confidence limits. Bold figures are either calculated net changes in SiO₂ (and one value for Na₂O), or upper limits for net change. Silica data show significant additions. Results for aluminium, sodium and potassium generally give the upper limit for net change (the quoted uncertainty).

TABLE 3 Petrographic point-count abundance of quartz (silica) cement

	Brae	Ula	Garn
Number of samples	56	36	26
Point counter	5.3 ± 3.2	3.2 ± 3.6	6.9 ± 5.6
Calculated from chemical composition	15.1 ± 7.1	8.7 ± 9.8	19.0 ± 8.4

All values are in %.

of the cored interval in the well (99.9% confidence limits). Selected samples included calcite-cemented concretions and host sandstone.

Similar results were obtained for all sandstones: they imported large and statistically significant quantities of silica during diagenesis. A worked example is given below for the Garn Formation (Table 1). Table 1 shows that a statistically significant quantity of silica was introduced into the sandstone during diagenesis, whereas some sodium was lost. The upper limits to changes in aluminium and potassium contents are given by their quoted uncertainties. Similar calculations for the four data sets are summarized in Table 2. We have used units of mass transferred (assuming a rock density of $2,700 \text{ kg m}^{-3}$) rather than percentage change, because it is independent of the porosity and thus of the initial silica content.

The best available independent test of the validity of the results is petrographic analysis of recognizable quartz cements. We used standard methods, and the comparisons are shown in Table 3. With the exception of the data for Brae, petrographic point-counter data give much lower values. We believe this is because most of the quartz cement occurs as syntaxial overgrowths on detrital grains and is difficult to identify.

We must next consider the possible source of the silica added to the reservoir sand unit. The most obvious external sources are mudrocks, which are the major component of sedimentary basins (65–92%)²⁰. There is ample evidence for the presence of silica in mudrocks, for reactions that could generate silica, for transfer-pathways and for transfer of material (but not silica) from mudrocks to sandstones. In a typical basin-fill, two lithologies, marginal sands and distal muds are often interdigitated. This would provide ready access for fluids moving into the sandstones.

The presence in sandstone of petroleum generated in mudrocks is proof that mudstones act as a source of some fluids. Aqueous fluids may follow similar pathways. The carbon isotope compositions of carbonate cements in a sandstone reservoir show that they result from degradation of abundant organic matter, more than could originally have been present in the sandstone²¹. The cementation was caused by aqueous fluids from an organic-rich (oil source) rock. Further confirmation of the relationship of mudrocks to silica-rich aqueous fluids is shown by fluid inclusions, the microscopic voids in minerals which may contain the fluid present at the time of mineral growth. Diagenetic quartz cements containing oil as well as brine have been reported²², indicating that oil was present when quartz precipitated from an aqueous fluid. Although there is no proof that the two fluids were cogenetic, it is plausible that they were. These observations imply that mudrocks and sands could be interactive parts of one system.

There is abundant silica in mudrocks, present both as the fine-grained quartz and as silicate minerals. Detrital smectitic clay will be transformed to illite during burial⁵, and the reaction has been shown²³ to release Si. (Many North Sea mudrocks at deposition were rich in smectite which has already undergone transformation to illite²⁴.) Although other sandstones could be sources of silica, all that we examined showed increased silica concentrations. Additionally, there is preliminary evidence for loss of silica from mudrocks during burial diagenesis, obtained by the same method that we used for sandstones²⁵.

This quantification of material flux challenges numerical models of fluid transport and ore emplacement²⁶, which relate driving force, permeability and flux in sedimentary basins. These models are plausible but are hard to test because duration of mineralization, and thus flux, is almost impossible to constrain. Observations that quartz cementation is a relatively rapid event (1 to 10 Myr)²⁷ constrain the model and produce higher than observed values for either flow rate or permeability. The relevant components of the process—solubility of silica, volumes of water involved and flow mechanisms—must be re-assessed. Preliminary calculations of the fluid volume available to be expelled from mudrocks during deep burial show that it is insufficient to transport enough silica in solution, even assuming considerable supersaturation^{3,28}. Any successful model of cementation of sandstone by silica must consider source, transportation and precipitation mechanisms. None of these is sufficiently quantified, and we are investigating all three. □

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Population variation in the ontogeny of predator-induced vertical migration of copepods

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DIEL vertical migrations (DVM) of zooplankton are nearly ubiquitous and vary widely in timing and magnitude^{1–5}. Predation can be both an evolutionary and proximal ('inducing') agent in promoting this variation^{6–14}. Because aquatic predators are strongly size-selective¹⁵, vulnerability to predation changes during an individual's ontogeny. Accordingly, natural selection on phototaxis, timing and magnitude of migration should also vary during ontogeny. Furthermore, biogeographic variation in the composition, density and behaviour of predators would probably select for different ontogenies of DVM. Whether predator-induced or

TABLE 1 Characteristics of the two adjacent (<0.5 km) montane lakes in coastal British Columbia (49° 19' N, 122° 34' W)

	Placid L.	Surprise L.
Elevation (m)	588	616
Area (ha)	0.8	0.7
Maximum depth (m)	6.5	7.2
Mean depth (m)	4.6	3.8
Organic stain (mg per litre Pt)	14–17	13–15
Secchi depth (m)	3.7–4.6	3.9–5.2
Chlorophyll <i>a</i> (μg per litre)	0.3–2.1	0.3–2.4
Phytoplankton (number ml^{-1})	130–565	115–455
Dominant phytoplankton	Chrysophyta Cryptophyta	Chrysophyta Cryptophyta
<i>Onchorhynchus clarki</i> (number)	<400	0
<i>Chaoborus flavicans</i> (number per m^3)	300–700	0
<i>Chaoborus trivittatus</i> (number per m^3)	0	50–240
Adult <i>Diaptomus kenai</i> (number per m^3)	630–1050	810–1460
Size of adult <i>D. kenai</i> (mm)	3.04 ± 0.14 (f) 2.88 ± 0.17 (m)	3.11 ± 0.19 (f) 2.97 ± 0.22 (m)
Other macrozooplankton (number per litre)		
<i>Daphnia rosea</i>	1.4–3.4	0.9–3.0
<i>Holopedium gibberum</i>	0.4–4.5	0.8–3.6
<i>Ceriodaphnia pulchella</i>	0.1–3.3	0.1–4.0
<i>Bosmina longirostris</i>	0.2–2.7	0.3–1.5
<i>Diaptomus oregonensis</i>	0.7–6.5	0.2–3.9
<i>Diacyclops thomasi</i>	3.8–9.7	2.6–8.1

developmentally fixed (canalized) migrations would be favoured probably depends on the variability of predatory risk^{16–19}. Here I compare experimentally DVMs of calanoid copepods from populations with contrasting histories of vertebrate and invertebrate predation. Results show predator- and population-specific migrations that differed with life history stage/size. Both developmentally fixed and predator-induced migrations were found at various life-stages in the same group of individuals. Ontogenetic patterns, however, differed among populations adapted to different suites of predators. Genes coding for differences in phenotypic expression of migratory behaviour seem to have been selected by local predation at each stage/size.

Migratory responses to each of three predators were compared in 10-m^3 field enclosures during ontogeny of the large, univoltine calanoid copepod *Diaptomus kenai* Wilson from two similar, adjacent lakes (Table 1). Experimentally manipulated predators included visually foraging juvenile cutthroat trout *Oncorhynchus clarki*²⁰ and small (12.5–14 mm) nocturnally planktivorous phantom midges *Chaoborus flavicans* from Placid Lake, and large (17–19 mm) *C. trivittatus* from fishless Surprise Lake (details in Table 2). Other rare or nonstratified predators in these two lakes, including *Diacyclops thomasi* (exclusively a predator of naupliar *D. kenai*²¹) were not manipulated because of their logistical intractability. The latter were found in all experimental enclosures. Copepods to be tested against these predators had no previous experience with any predators. Isolation from potential predators before testing prevented selective predatory mortality, and so cohort genetic change by predation, during postembryonic development. It also allowed expression of inducible migrations (phenotypic plasticity) only in experimental enclosures. Rapid induction (<8 h after predator additions) and maintenance of new migratory patterns, relative to nonpredation controls, indicated behavioural sensitivity to that predator.

Nauplii in both lakes, all control and predation enclosures (Table 2), and all predator-free rearing enclosures were non-migratory and invariantly surface oriented (98% <1.5-m deep). Fish stomachs contained no nauplii, but included 265–410 cladocerans (*Daphnia* and *Holopedium*) and 25–55 adult cyclopoid copepods. Small and large chaoborid species consumed 3.8–6.6 nauplii per crop, as well as 0.2–1.1 immature cyclopoid copepods and 0.3–1.4 *Daphnia*, *Ceriodaphnia* and *Bosmina*. No naupliar migrations were observed in any enclosure at the beginning or end of the trial (day 1 and 14).

In contrast to unresponsive nauplii, identical, 'reverse' vertical migrations were rapidly induced (<7 h) by each chaoborid

species in both small (stages C1–C3, 0.6–1.5 mm) and large (C5–C6, 2.5–3.3 mm) copepodid stages from fishless Surprise L. (Fig. 1, Table 2). Migrating copepodids descended through the mass of ascending chaoborids at dusk, and returned to upper strata near dawn. Trout had no effect on DVM during any copepodid stages that, like those in controls, remained continuously above 3-m depth (Fig. 1). Migration patterns expressed during the first 24 h in all enclosures persisted until the end of each trial (day 30). About 6.1% of large *C. trivittatus* and 4.0% of smaller *C. flavicans* captured at least one newly migrating C1–C3 copepodid per day. Newly migrating C5–C6 copepodids had lower mortality (2.4% of *C. trivittatus* and 0.0% of *C. flavicans*). *Bosmina*, small *Daphnia*, *Diacyclops*, and *Diaptomus oregonensis* comprised most of their diets. Fish ate few C1–C3 copepodids (5.1 per stomach; $n=6$), concentrating on more common *Daphnia* and *Holopedium* (range: 282–506 per stomach, 1.1 mm mean length). By contrast, trout preyed heavily (199–399 copepods per stomach) on large, nonmigrating C5–C6 copepods, as well as *Daphnia*, *Holopedium* and *Diacyclops*. In consequence, nonmigrating copepods in fish enclosures were exterminated (<0.02 l^{-1}) in 19–25 days. None of the other taxa in the enclosures showed consistent vertical migration in the presence of predators.

Unlike the Surprise L. stock, the timing, magnitude and predator-inducibility of DVM changed as copepods from Placid L. matured (Table 2; Fig. 2). Small, unenclosed C1–C3 copepodids were reverse migrators in the lake, but showed no migrations in controls and trout enclosures. Reverse migrations reappeared

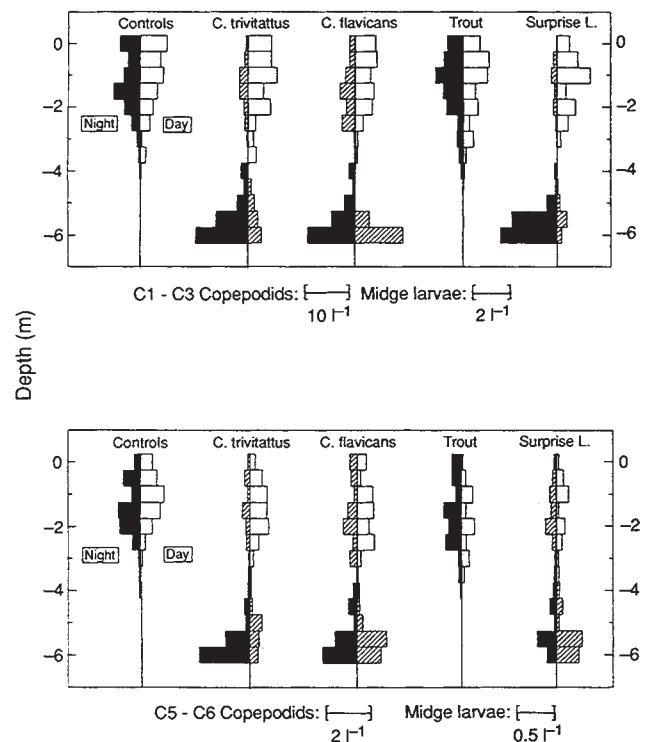


FIG. 1 Average depth-density profiles of predator-naive *D. kenai* (white or black bars) from Surprise Lake stock exposed to separate predation treatments in triplicate enclosures installed in Placid L. The predator added to each treatment is indicated above each histogram. Nighttime depths (24:00 $\pm$ 1 h) of copepods and chaoborids (hatched bars) are on the left side of each histogram, and daytime depths (13:00 $\pm$ 1.5 h) are shown on the right. Controls contained no added predators. Free-ranging copepods and chaoborids in Surprise Lake are shown at far right of each panel. Tested copepods had been reared from eggs in predator-free enclosures before exposure to predators. Histograms describe spatial distributions within 8 h (night sample) and 20 h (day sample) of predator additions to enclosures. Upper panel, C1–C3 copepodids; lower panel, C5–C6 copepodids.

<6 h after experimental addition of either chaoborid. Small copepodids occurred in 3.5–5.7% of chaoborid crops and averaged 3.4 per stomach in fish. By contrast, large C5–C6 copepodids were invariant 'nocturnal' migrators (night-time ascent <3 m, day-time descent >5 m). Regardless of predation test regime, apparently obligate nocturnal migration occurred in all 12 experimental enclosures, including controls (Fig. 2), and in all four 8-month-old zooplankton-free rearing enclosures. (I have recently confirmed invariant nocturnal DVM in large copepodids from this population using naive, laboratory-reared individuals tested in 2-m-high columns filled with autoclaved tap water.) Nocturnally migrating copepodids from Placid L. had low mortality from trout (4.2 per stomach) and small *C. flavicans* (<0.2% of crops). But when exposed to nocturnally migrating *C. trivittatus*, their mortality was high (61.4% of crops). During the 30-day trial, reverse migrations were never readopted in any chaoborid enclosure and all were exterminated by large midge larvae in 18–27 days. Thus, in contrast to Surprise L. copepodids, maturing copepodids from Placid L. appear to lose previous responsiveness to chaoborids in favour of seemingly invariant nocturnal DVM.

These results indicate that DVM during each copepodid life stage is locally adapted to the size/stage-selective predators normally preying on that stage. Timing, phototaxis and transit distances can change during ontogeny, correlated with changes in vulnerability. Surprise L. copepodids, which are potential prey of large, nocturnal *Chaoborus trivittatus* (but not fish) all their lives, exhibit 'reverse' (photopositive) DVM through adulthood. Few or none have the potential (negative phototaxis) to avoid exotic, diurnally foraging fish. By contrast, copepodids from adjacent Placid L. lose their photopositive avoidance of chaoborids and become photonegative when size/stage-dependent vulnerability shifts from small *C. flavicans* to visually foraging fish. Whether size/stage-dependent activation of genes for negative phototaxis inactivates those for positive phototaxis, or merely masks their expression, is unknown.

TABLE 2 Mean transit distances for *D. kenai*

Stage/lake	Control	Test predators		
		<i>Oncorhynchus clarki</i>	<i>Chaoborus trivittatus</i>	<i>Chaoborus flavicans</i>
Nauplii				
Surprise L.	0.11 (0.21)	0.07 (0.28)	0.13 (0.17)	-0.05 (0.10)
Placid L.	-0.08 (0.13)	-0.06 (0.18)	-0.19 (0.22)	0.13 (0.13)
Copepodid 1–3				
Surprise L.	-0.06 (0.30)	-0.22 (0.17)	-4.29 (0.33)	-4.53 (0.19)
Placid L.	-0.27 (0.16)	-0.19 (0.22)	-4.88 (0.40)	-4.38 (0.50)
Copepodid 5–6				
Surprise L.	-0.23 (0.32)	0.08 (0.54)	-4.22 (0.37)	-4.32 (0.54)
Placid L.	3.97 (0.39)	4.19 (0.39)	3.84 (0.43)	3.80 (0.36)

Mean transit distances (calculated from density-weighted day depth – night depth) in metres (± 1 s.d.) for three life-history stages of *D. kenai* under four predatory conditions in enclosures in Placid L. Enclosures were 1.55-m diameter $\times$ 6 m polyethylene plastic tubes¹⁰ filled with Placid L. water and plankton from which resident predators and *D. kenai* had been removed by sieves and hand sorting²⁴. Enclosures were permitted to equilibrate for 7–10 days before stocking with nauplii, copepodid instars C1–C3, or instars C5–C6 (adult) of *D. kenai*. All stocked copepodids had been reared in otherwise zooplankton-free enclosures (71–83% survival) from resting eggs collected from Placid and Surprise Lakes. Natural densities of fourth instar phantom midges (500 m⁻³ *Chaoborus flavicans*, 12.5–14 mm, or 150 m⁻³ *C. trivittatus*, 17–19 mm), or two 60–70-mm 1+ juvenile trout from Placid Lake were added to each enclosure 4–6 h after copepodids had been added. Controls lacked predators. Duplicate 75-l pump samples were taken at 0.5-m depth intervals to document spatial distributions within 8 h (night sample) and 20 h (day sample) of predator introductions to enclosures. Trials against each predator ran for 14–30 days (sampled 1–3 times during each trial) to assess stability of initial migratory responses and measure demographic impacts (reported elsewhere). All test copepodids were discarded after each run. Statistically indistinguishable transit distances (Tukey HSD tests after 3-way anova on log transformed depths, $\alpha=0.01$) are shown inside boxes. $n=3$ enclosures.

These results also indicate that both predator-induced and noninduced migrations may occur during an individual's development. Copepodid instars C1–C3 from Placid L. and C1–C6 from Surprise L. are capable of rapidly expressing reverse DVM when chaoborids are chemically¹⁰ detected. Nauplii from both lakes and copepodids C5–C6 from Placid L. are behaviourally unresponsive to any predator tested. Natural selection for relatively 'closed'²² (inflexible) expression of genes involved in predatory defence is predicted when risk from predators is relatively invariant^{18,19}. Risks for nauplii and large copepodids in Placid L. probably are relatively constant. Population densities of *Diaicyclops thomasi*, the principal naupliar predator, and cutthroat trout in Placid L. are stable between years and continuously food-limited^{20,21}. By contrast, when net fitness benefits of defences vary unpredictably within and across a few individual lifetimes, reaction norms of those genes are predicted to be large^{17,19}. Long-lived copepodids like *D. kenai* are especially susceptible to variability in densities of invertebrate predators. As with all other known predators that also induce DVM in zooplankton^{6–14}, very variable recruitment^{11,14}, survival^{10,23} and/or spatial distributions^{8,9} characterize third and fourth instar *Chaoborus* populations in these small lakes (densities vary interannually from 0–1,100 m⁻³). The relative predictability of risk of mortality from these two groups of predators seems to favour differences in ontogenic expression of genes regulating antipredator migrations. □

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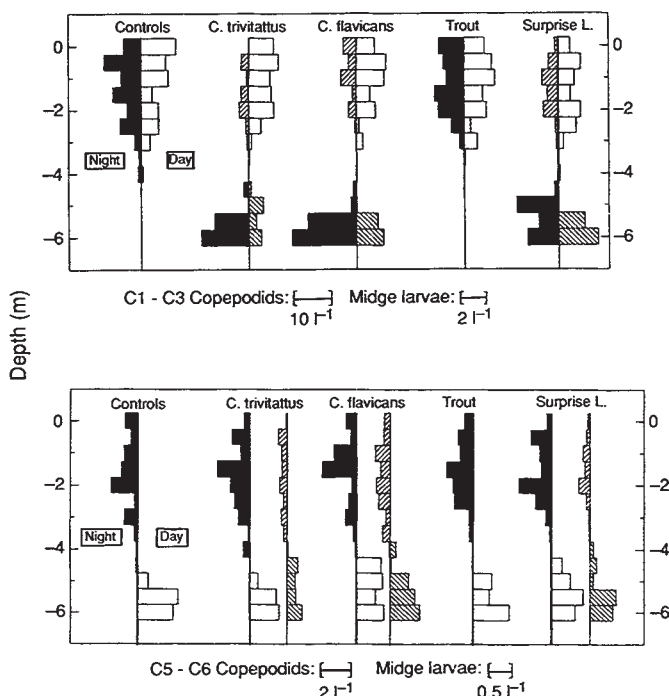


FIG. 2 Average depth-density profiles of predator-naive *D. kenai* (white or black bars) from Placid Lake stock exposed to separate predation treatments in triplicate enclosures installed in Placid L. Profiles of copepodids and chaoborids are shown separately for each treatment to clarify presentation. See legend to Fig. 1 for details and labelling conventions.

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A chloride channel widely expressed in epithelial and non-epithelial cells

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CHLORIDE channels have several functions, including the regulation of cell volume^{1,2}, stabilizing membrane potential^{3,4}, signal transduction^{5,6} and transepithelial transport⁷. The plasma membrane Cl^- channels already cloned belong to different structural classes: ligand-gated channels^{5,6}, voltage-gated channels^{8,9}, and possibly transporters of the ATP-binding-cassette type (if the cystic fibrosis transmembrane regulator¹⁰ is a Cl^- channel^{11–13}). The importance of chloride channels is illustrated by the phenotypes that can result from their malfunction: cystic fibrosis, in which transepithelial transport is impaired, and myotonia³, in which ClC-1 , the principal skeletal muscle Cl^- channel, is defective⁹. Here we report the properties of ClC-2 , a new member of the voltage-gated Cl^- channel family. Its sequence is ~50% identical to either the *Torpedo* electroplax Cl^- channel, ClC-0 (ref. 8), or the rat muscle Cl^- channel, ClC-1 (ref. 9). Isolated initially from rat heart and brain, it is also expressed in pancreas, lung and liver, for example, and in pure cell lines of fibroblastic, neuronal, and epithelial origin, including tissues and cells affected by cystic fibrosis. Expression in *Xenopus* oocytes induces Cl^- currents that activate slowly upon hyperpolarization and display a linear instantaneous current–voltage relationship. The conductivity sequence is $\text{Cl}^- \geq \text{Br}^- > \text{I}^-$. The presence of ClC-2 in such different cell types contrasts with the highly specialized expression of ClC-1 (ref. 9) and also with the cloned cation channels, and suggests that its function is important for most cells.

A complementary DNA encoding the rat skeletal muscle Cl^- channel ClC-1 (ref. 9) was used to screen cDNA libraries from rat heart and brain under relaxed stringency. Sequencing of positive clones indicated the presence of novel Cl^- channel (ClC-2) messenger RNAs that were identical in both tissues. Clones were more abundant in the brain library, from which several large (>3 kilobases (kb)) clones were isolated, the largest of which was fully sequenced (Fig. 2). The putative initiator methionine is followed by a hydrophobic stretch of ~11 amino acids, a feature not found in other channels^{8,9} of this family. The sequence predicts a 907-amino-acid protein of relative molecular mass ~99,000 having a transmembrane topology similar to that of related Cl^- channels^{8,9}. Overall homology to the *Torpedo* channel ClC-0 is 49%, and 55% to the rat muscle

Cl^- channel ClC-1 . Divergence is greatest at the N- and C-termini and between domains D12 and D13.

Northern analysis revealed that ClC-2 is expressed in all the tissues that we examined (Fig. 1a). In addition to a common mRNA of ~3.3 kb, larger species (~4.5 kb) were detected in some tissues (for example, kidney and brain) and may represent splice or polyadenylation variants. Established cell lines were used to investigate expression in defined cell types (Fig. 1b). The ~3.3-kb transcript was found in all these cell lines, with the additional ~4.5-kb band being detected primarily in human and monkey cells. ClC-2 is thus transcribed in typical fibroblast (3T3) and epithelial cells (T84; a human colon cell line¹⁴, CFPAC-1; a human pancreatic cystic fibrosis cell line¹⁵, and LLC-PK₁), as well as in neuronal-like cells (Neuro-2a, PC-12). In some tissues and cells (for example, heart and skeletal muscle, A10) ClC-2 is co-expressed with ClC-1 (ref. 9). It will be interesting to see if these homologous proteins form hetero-oligomeric channels with new properties.

In *Xenopus* oocytes, Cl^- currents could not be expressed from

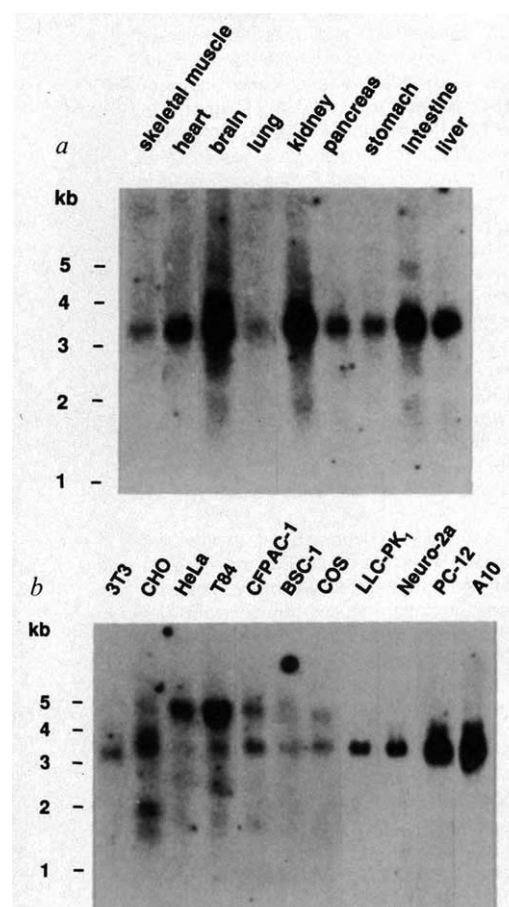


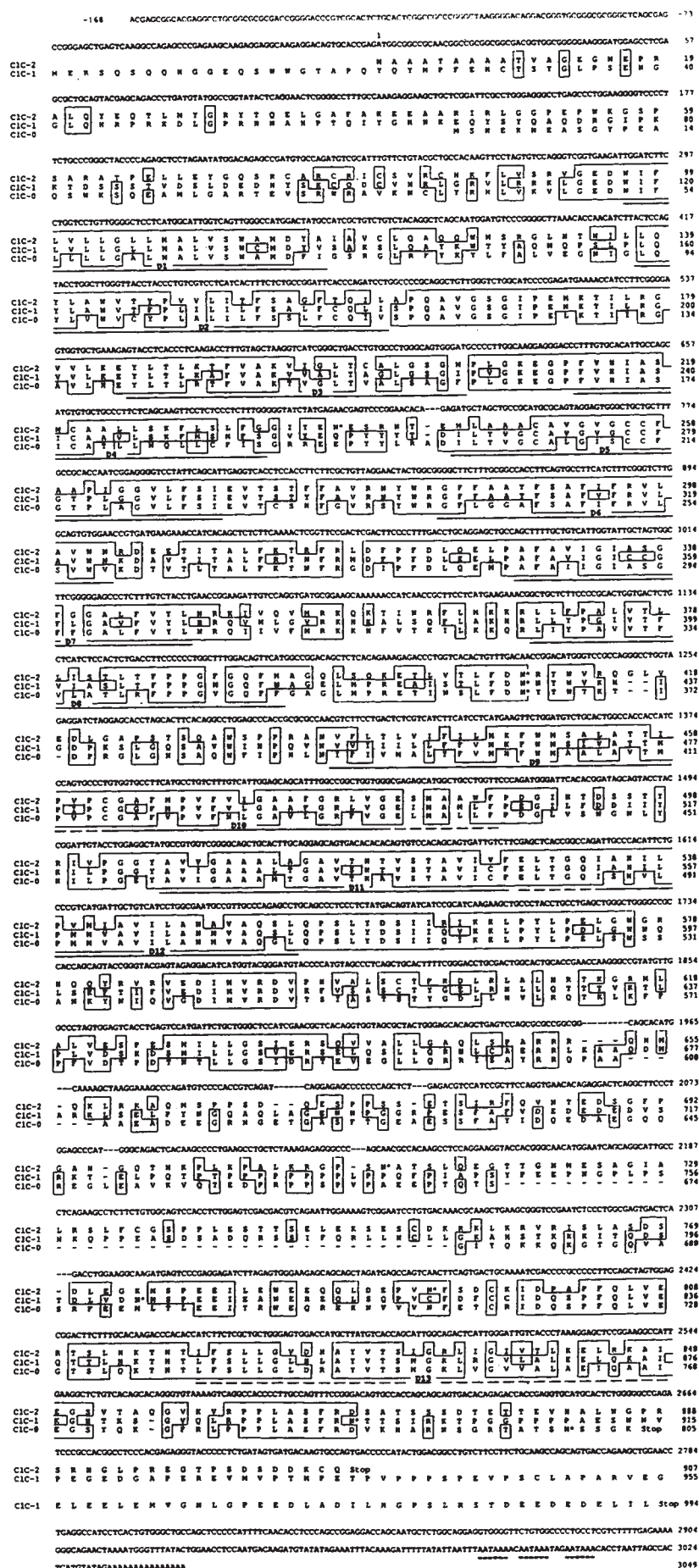
FIG. 1 Northern analysis of ClC-2 expression. *a*, Expression in different rat tissues; *b*, expression in established cell lines.

METHODS. RNA was extracted from several tissues and cell lines and enriched for poly(A)⁺ tracts: 10 µg each (by absorbance at 260 nm) (15 µg for Neuro-2a and LLC-PK₁) were run on agarose gels containing formaldehyde. Equal loading and absence of degradation was additionally checked by staining with ethidium bromide. The size standard is a ³²P-labelled 1-kb DNA ladder (BRL). After transfer to nylon membranes, blots were hybridized under high stringency with clone λb12-2 labelled with ³²P, and autoradiographed. Cell lines are: 3T3, NIH3T3 mouse fibroblasts; A10, rat aorta smooth muscle (CRL1476); BSC-1, African green monkey kidney (CCL26); CFPAC-1, human pancreatic tumour expressing the CF phenotype¹⁵; CHO, CHO-K1 Chinese hamster ovary cells (CCL61); COS, COS-7 SV40 transformed monkey kidney (CRL1651); HeLa, human epitheloid cervix carcinoma (CCL2); LLC-PK₁, pig kidney (CL101); Neuro-2a, mouse neuroblastoma (CCL131); PC-12, rat adrenal pheochromocytoma (CRL1721); T84, human colon carcinoma (CCL248). Culture conditions were as recommended for each specific line.

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FIG. 2 Primary structure of chloride channel CIC-2. Nucleotide sequence of CIC-2 and alignment of its deduced amino-acid sequence with that of the rat skeletal muscle Cl^- channel CIC-1 (ref. 9) and the *Torpedo* channel CIC-0 (ref. 8). Conserved residues are boxed, putative transmembrane domains³⁰ underlined, and potential N-linked glycosylation sites indicated by asterisks. A highly conserved glycosylation site is present between D8 and D9, and additional ones at positions 238, 712 and 794. Polyadenylation signals are double-underlined. The initiator methionine was assigned to the first ATG preceding a long open reading frame. This ATG, as well as a second one following 45 base pairs (bp) downstream in frame, is surrounded by sequence fulfilling the requirements for eukaryotic initiation³¹. As there is no upstream stop codon in frame, we cannot be absolutely sure whether this represents the authentic initiator ATG. But we cannot miss much upstream sequence (length of cDNA, excluding the poly(A) tail, is 3,202 bp, versus a message size of ~3.3 kb by northern analysis (Fig. 1)), and one independent clone terminated within 5 bp, and 2 clones within 150 bp of the shown 5' end. Moreover, functional Cl^- channels could be expressed starting from this ATG (Fig. 3).

METHODS. An oligo(dT) primed rat brain cDNA library in λ ZAPII (a gift from S. Morley and W. Meyerhof) and a rat heart cDNA library (Clontech) were screened under reduced stringency (in $5 \times \text{SSC}$, $5 \times \text{Denhardt's}$ solution, 25% formamide, 0.1% SDS at 42°C) with a radiolabelled CIC-1 (ref. 9) cDNA probe (extending from bp 990 to 1,881). Positive clones were confirmed by sequencing and used to rescreen the brain library at high stringency. The sequence shown derives from clone λ b12-2 (except for the poly(A) tail, which derives from clone λ b9), which was fully sequenced on both strands using T7 DNA polymerase in the chain termination method. The sequence has been deposited in the EMBL/GenBank database under accession number X64139.



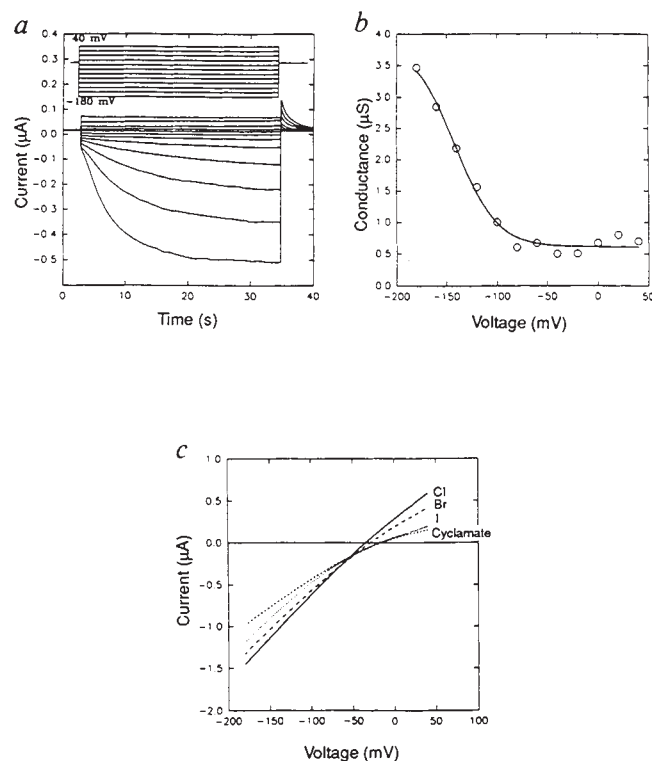


FIG. 3 Electrophysiological analysis of CIC-2 expressed in *Xenopus* oocytes. **a**, Voltage-clamp traces. Voltage was clamped for 32 s from a holding potential of -30 mV to values between -180 and $+40$ mV in 20 mV steps (inset), and the current necessary to clamp the oocyte was recorded. Note that currents activate slowly upon hyperpolarization, and that deactivation is much faster. **b**, Quasi-steady-state conductance as a function of voltage (measured after clamping for 32 s to the indicated potential); reversal potential was assumed to be -30 mV. The curve fitted is of the form $G(V) = G_0 + G_1 / (1 + \exp((V - V_0)/K))$. Note that conductance between $+40$ and -80 mV is constant and similar to that of control oocytes, suggesting that the vast majority of CIC-2 channels is not open. Conductance increases steeply at voltages more negative than -100 mV without saturating at -180 mV. Mean conductance at -180 mV (after at least 30 s) was 8.2 ± 0.8 (s.e.m., $n=12$) μ S for cRNA-injected oocytes, and 2.2 ± 0.5 (s.e.m., $n=9$) μ S for non-injected controls. In the range between 0 and 20 mV, conductance averaged to 1.4 ± 0.2 (s.e.m., $n=12$) μ S for injected, and 2.9 ± 0.9 (s.e.m., $n=9$) μ S for control oocytes. **c**, Instantaneous current-voltage (I - V) relationships of activated channels in the presence of different extracellular anions (from a different oocyte). Immediately after activation by hyperpolarization (-180 mV for 32 s), fast voltage ramps (0.5 s) in positive or negative directions were used to minimize effects of deactivation. Results were not significantly different from those obtained by tail-current analysis. Ion selectivity was tested by partially replacing 80 mM extracellular chloride with iodide, bromide, or cycamate. Data were not corrected for liquid junction potentials. The reversal potential ($I=0$) shifts towards positive voltages on chloride removal, indicating a chloride-selective channel. Note that the I - V curve for Cl^- is nearly linear, and that the channel displays a $\text{Cl}^- \gg \text{Br}^- > \text{I}^-$ selectivity sequence.

METHODS. Recombinant polymerase chain reaction (PCR)³² was used to construct plasmid ptm1-b12-2 in which 83 bp of $5'$ sequence immediately preceding the initiator ATG of CIC-0 (ref. 8) was placed in front of the first ATG of CIC-2 clone lb12-2. The resulting chimaeric PCR product was digested with *Xho*I and ligated at the 54 -bp *Xho*I site to clone lb12-2. The sequence of the new fragment was fully verified. This does not introduce any new amino acids into the resulting protein. After linearization of the construct, capped RNA was synthesized *in vitro* using T7 RNA polymerase, about 50 ng of which was injected into *Xenopus* oocytes prepared and handled as described³³. They were stored and analysed in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, pH 7.6), or in ND96, in which 80 mM NaCl was replaced by 80 mM NaI, NaBr, or sodium cycamate. Electrophysiological analysis was at room temperature 2 – 4 days after injection using standard 2-electrode-voltage clamp technique and pCLAMP software.

the intact, putative full-length cDNA. We replaced sequence $5'$ to the first ATG by $5'$ non-translated sequence from CIC-0, a previously successful strategy⁹. Expression of derived cRNA in *Xenopus* oocytes led to Cl^- currents that were not detectable under resting conditions, but increased slowly (within tens of seconds) after hyperpolarization. An activation by hyperpolarization is also found with CIC-0 (refs 8, 16, 17), but not with CIC-1 (ref. 9). With CIC-2, activation began at about -90 mV and did not saturate even at -180 mV (Fig. 3a, b). Stepping back to the holding potential (-30 mV) caused deactivation within seconds. Instantaneous I - V curves of activated channels revealed a linear current-voltage relationship (Fig. 3c). Partial replacement of extracellular chloride is indicative of an anion-selective channel with a $\text{Cl}^- \gg \text{Br}^- > \text{I}^-$ conductivity sequence. Although iodide blocks CIC-0 (refs 16, 17), it permeates CIC-2. Extracellular 9-anthracene carboxylic acid (1 mM) and diphenylaminecarboxylate (1 mM), which are Cl^- channel inhibitors but also have other effects¹⁸, partially ($\sim 50\%$) inhibited the conductance, whereas 1 mM DIDS (4,4'-diisothiocyanato-2,2'-disulphonic acid) was almost ineffective (data not shown).

As CIC-2 is broadly expressed in different tissues and in cells of such varied origin as epithelial, fibroblast and neuronal cells, it may share some important housekeeping role in these cells. The channel is closed at physiological voltages, so perhaps some other mechanism of activation (in addition to hyperpolarization) exists *in vivo*. The expression of CIC-2 in T84 epithelial cells is especially interesting and was verified by cloning partial T84 CIC-2 cDNAs (unpublished results). T84 cells have served as a model system for Cl^- transport, highly relevant for cystic fibrosis (CF). They express at least four different Cl^- channels, three of which conduct iodide better than chloride and are outward-rectifying (volume-sensitive^{1,19} and Ca^{2+} -activated^{20–22} channels, and an obscure^{20,23} 40 – 60 -pS channel, formerly thought to be affected in CF (for review, see ref. 7)). The fourth channel has a time-independent linear I - V relationship, a $\text{Cl}^- > \text{I}^-$ selectivity, and probably mediates the cyclic AMP-activated current defective in CF (refs 20, 21, 24). On the basis of the instantaneous I - V relationship, selectivity and pharmacology, CIC-2 most closely resembles this latter channel. It is unclear, however, whether the channel affected in CF can be activated by strong hyperpolarization, and whether CIC-2 can somehow interact with the cystic fibrosis transmembrane regulator (CFTR)¹⁰, the CF gene product.

CFTR, previously thought to regulate Cl^- channels²⁵, is now believed to be a cAMP-activated channel itself. This is based on two main findings: first, CFTR expression in different cells (for example, CFPAC-1 (ref. 26), 3T3 (ref. 11), HeLa (refs 11, 27), CHO (refs 11, 28), T84 (ref. 11), Sf9 (ref. 12)) induces cAMP-activated Cl^- currents. It was unlikely that all these cells would have pre-existent similar Cl^- channels serving as targets for CFTR. Our work nearly invalidates this argument, though we did not test non-mammalian cells because of expected difficulties in cross-species hybridization. Second, and most convincing, mutagenesis of CFTR changes ion selectivity¹³ and cAMP-dependence^{27,29} of currents. Thus, certain similarities of CIC-2 with the Cl^- channel affected in CF are tantalizing, but probably coincidence. The functions of CIC-2 remain to be identified, but these are important because it is expressed in cells affected by CF. □

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Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells

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In synapses, a rise in presynaptic intracellular calcium leads to secretory vesicle fusion in less than a millisecond, as indicated by the short delay from excitation to postsynaptic signal^{1–4}. In non-synaptic secretory cells, studies at high time resolution have been limited by the lack of a detector as fast and sensitive as the postsynaptic membrane. Electrochemical methods may be sensitive enough to detect catecholamines released from single vesicles^{5,6}. Here, we show that under voltage-clamp conditions, stochastically occurring signals can be recorded from adrenal chromaffin cells using a carbon-fibre electrode as an electrochemical detector. These signals obey statistics characteristic for quantal release; however, in contrast to neuronal transmitter release, secretion occurs with a significant delay after short step depolarizations. Furthermore, we identify a pedestal or 'foot' at the onset of unitary events which may represent the slow leak of catecholamine molecules out of a narrow 'fusion pore' before the pore dilates for complete exocytosis.

Isolated bovine chromaffin cells were used to study catecholamine release under patch-clamp whole-cell recording conditions. We applied short step depolarizations to open voltage-gated calcium channels⁷, thereby injecting calcium to activate secretion⁸. When the tip of a carbon-fibre electrode^{5,9,10} was placed less than 1 µm from the cell to record in amperometric mode, spike-like current transients were seen at variable times after the onset of depolarizing pulses (see Fig. 1a). These signals occurred rarely or not at all, unless the cell had been stimulated; on stimulation they appeared with a frequency that peaked shortly after the voltage step and that subsequently decayed over about 100 ms. Furthermore, they were seen only if the potential applied to the carbon-fibre electrode was greater than about 250 mV (exceeding the oxida-

TABLE 1 Single secretory events follow a Poisson distribution

Cells	n_0	n_1	n_2	n_3	$n_{>3}$	N	events
A							
Observed	45	52	26	7	3	133	139
Calc. ($m=1.01$)	47	49	26	9	2		
B							
Observed	8	18	17	12	10	65	133
Calc. ($m=2.05$)	8	17	18	12	10		
C							
Observed	15	28	20	8	3	74	105
Calc. ($m=1.42$)	18	25	18	9	4		
D							
Observed	28	19	11	0	0	58	41
Calc. ($m=0.71$)	29	20	7	2	0		

Three cells with a low probability of release were selected for analysis. Cells A, B, C and D were repetitively stimulated every 5 s by depolarizing pulses to +10 mV for 25 ms (A, C) or 50 ms (B, D). D was, in fact, the same cell as C, but at a much later time in the experiment, when the release probability had decreased. Each discernible current transient, regardless of size, was counted as one event. $n_{0,1,2,3,\dots}$: numbers of times that pulses elicited 0, 1, 2, 3, ..., x events during the 5-s interval after a pulse. Calculated numbers: from Poisson's law

$$P_x = n_x/N = e^{-m} m^x / x!$$

N , Total number of step depolarizations.

m , (total number of events)/ $N = (n_1 + 2n_2 + 3n_3 + \dots + xn_x)/N$.

tion potential for catecholamines). When the detector was moved several microns away from the cell, the signals became smaller and slower. Stimulating with longer depolarizations generally led to an increase in the number and frequency of unitary events, not to an increase in their individual size (Fig. 1b). These observations suggest that the signals represent packages of oxidizable substance being secreted from the cell, in agreement with a previous report on unclamped cells⁵.

Another way to monitor secretion from single cells is to record the membrane capacitance^{8,11}. This electrical parameter is proportional to cell surface area and increases when vesicles fuse with the plasma membrane during exocytosis. When a cell was stimulated repetitively (Fig. 2), the time-averaged signal of the carbon-fibre electrode closely resembled the derivative of the capacitance trace, thus confirming that both quantities reflect rate of transmitter release. In another cell (data not shown), short depolarizing pulses each elicited an average of 1.05 unitary events and a 13-fF increment in capacitance. Assuming that each event is due to a single vesicle and that each vesicle contributes 2.5 fF (as suggested from previous measurements⁸ and from morphometry), we conclude that the carbon-fibre electrode detected about 15–25% of the quanta released. This compares well with the fraction of the cell surface area in closest apposition to the detector surface (see Fig. 1 legend).

Amperometric currents from secreting chromaffin cells arise almost exclusively from oxidation of catecholamine molecules⁶, and each molecule contributes two electronic charges¹². Thus, one can estimate the number of catecholamine molecules per transient from the total charge of the unitary signal. Figure 1c shows a histogram of the integrals of the current transients from three cells. From its mean the number of molecules detected can be calculated as 2.4–3.2 million (see Fig. 1 legend), agreeing with previous estimates (3×10^6 molecules) for single bovine chromaffin granules¹³.

For synapses the number of vesicles released per stimulus follows binomial or Poisson statistics¹. As shown in Table 1, such statistics also apply to secretion from chromaffin cells. Although the unitary events are not as uniform as miniature synaptic currents, they are generally well separated in time (for submaximal stimuli), making it possible to count them directly (something not possible for postsynaptic signals except at very low temperatures and low calcium¹⁴).

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Unlike the neuromuscular junction¹, the squid giant synapse^{2,3} or neurons⁴, for which synaptic events occur about 1 ms after stimulation, the latency in chromaffin cells is quite long at 51 ± 7.3 ms (see Fig. 1d for an example of a latency histogram). Use of the nystatin method¹⁵ to minimize perturbations of the cytoplasmic constituents did not reduce the latency, ruling out that 'washout' of intracellular mediators artificially prolonged the response time.

When examining specifically single events with fast rise time (events which should report the time course of release most faithfully), we observe something surprising: most of the signals have a small 'foot' or 'pedestal' preceding an abrupt upstroke (see Figs 3 and 1a for examples). One possible explanation could be the near-simultaneous release of two vesicles. At least

two findings rule out such a hypothesis, however, unless a very strong, time-dependent correlation between the two release events is postulated (see legend to Fig. 3). First, the mean time between the 'foot' event and the main event is much shorter than the time interval expected for two independent events on the basis of the latency distribution. Second, the mean amplitude of the foot event is much smaller than the overall mean. The latter finding in particular makes the hypothesis of superposition very unlikely. One would have to postulate that small events (expected to occur far from the recording carbon electrode) routinely precede large events (which probably occur close to the electrode). Although such a possibility cannot be totally excluded yet, we favour an interpretation in which pedestal and upstroke result from the same fusion event. If so, a likely

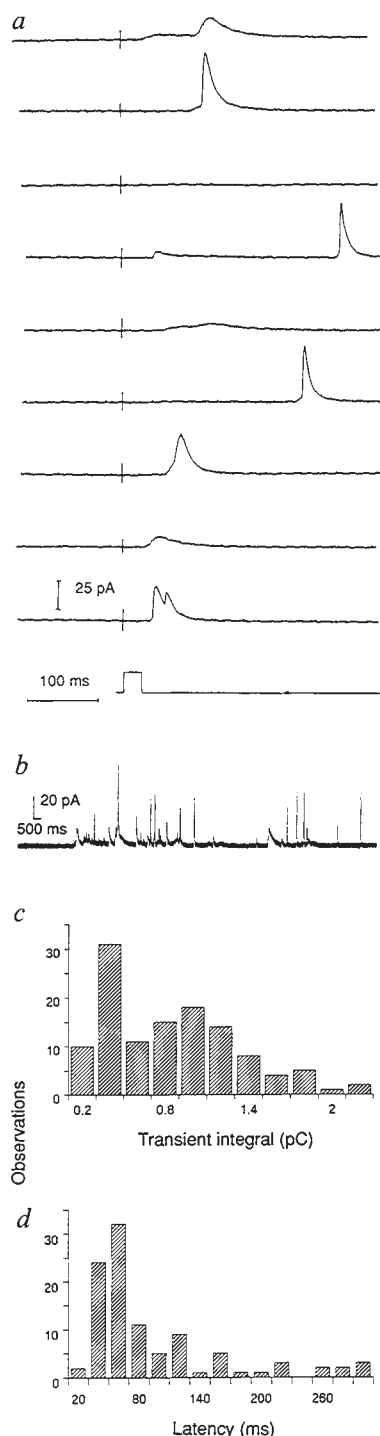


FIG. 1 Electrochemical approach detects secretion from single vesicles. *a*, Examples of signals recorded by the amperometric constant-voltage method. A cell (diameter $\sim 15 \mu\text{m}$) in the whole-cell patch-clamp mode was held at -60 mV and repetitively depolarized to $+10$ mV for 25 ms to activate Ca^{2+} channels. The tip of a carbon-fibre electrode (diameter $8 \mu\text{m}$), located $<1 \mu\text{m}$ from the cell (with the voltage held constant at 800 mV), recorded the evoked secretory signals. If the fibre tip detects molecules released from the cell surface that lies within the projection of a $12\text{-}\mu\text{m}$ disc, we estimate that about 15% of all events should be detected. The voltage protocol is shown in the bottom trace, and representative amperometric current signals are shown above, aligned in time relative to the stimulating pulse. The signals vary in amplitude and duration, and occasionally there are 'failures' (sweeps without a response). *b*, Response to steady-state depolarization to 0 mV after the recording of part *a* (note difference in time scales). *c*, Histogram of integrals of current transients in picocoulombs (pC). For 3 cells, all current transients having a fast rise time (<3 ms) were selected, because these signals presumably arise from vesicles opening closest to the detector. Time integrals of 84 current transients were plotted as a histogram. One extra large event is not included because it is off the scale (at 3.4 pC). In some other experiments, particularly after prolonged stimulation, such extra large events were more numerous. They may represent multigranular exocytosis²². The charge per transient (mean $\pm$ s.d.) is 0.76 ± 0.55 pC, equivalent to $2.36 (\pm 1.71) \times 10^6$ molecules of catecholamine. The histogram includes small events with short rise times which were probably due to vesicles opening close to the detector, but near its rim (where some molecules may diffuse away and escape detection). Excluding such small and fast 'rim' events by setting an amplitude threshold (20 pA), gives a mean charge of 1.00 ± 0.53 pC or $3.15 (\pm 2.61) \times 10^6$ molecules. *d*, Histogram of latency times (the time between beginning of the depolarizing step and the onset of the current transient). Release probability is high during the first 100 ms, and then drops to a lower but maintained value, which, at a compressed timescale, appears as another slow component with a decay time in the range of seconds. Such slow components were present in different cells to various degrees (comprising 23–53% of all events). In 4 cells the mean latency of the fast component was 51 ± 7.3 ms (mean $\pm$ s.e.m.).

METHODS. Bovine chromaffin cells were prepared as previously described²³ and kept for 1–5 days in short-term culture. Voltage-clamp control of membrane potential was achieved using the whole-cell patch-clamp method²⁴ or else the nystatin method¹⁵. Extracellular solution in mM: 120 NaCl; 2 CaCl_2 ; 2 MgCl_2 ; 10 Na-HEPES; 50 glucose; pH 7.2. Pipette solution: 145 Cs-glutamate; 8 NaCl; 1 MgCl_2 ; 2 Mg-ATP; 0.3 GTP; 10 Na-HEPES; pH 7.2; 0.1 Fura-2. Amperometric constant-voltage measurements were done as described^{5,6}, with some modifications in the method of preparation of the electrodes²⁵. A 4- to 5-cm length of $8\text{-}\mu\text{m}$ diameter carbon fibre was cannulated into 0.28 mm inner diameter polyethylene tubing, the middle of which was subsequently heated over a hot soldering iron tip. When melted, the tubing was pulled gently apart 1–2 cm, resulting in a narrowed region where the melted plastic formed an electrically tight seal with the carbon fibre. The narrowed region was cut by scissors, resulting in two electrodes with carbon exposed only at the very tips. Each such assembly was glued in a glass capillary tube, which was then back-filled with 3 M KCl solution. Electrical connection to an EPC-7 patch-clamp headstage was established through an Ag/AgCl wire inserted into the KCl solution. During experiments, the voltage of the electrode was set to 800 mV and the tip of the carbon-fibre electrode pushed gently against the cell. The oxidation currents were filtered in two stages at 10 kHz and 3 kHz, digitized and stored on a video cassette recorder. Experiments were done at room temperature ($22\text{--}30^\circ\text{C}$).

FIG. 2 Correlation between the amperometric signal and the rate of capacitance change. A cell was held at -60 mV and repetitively depolarized to 0 mV for about 8 s at a time (see lowest trace for voltage protocol). Capacitance (second trace from bottom) increased during depolarizations, and the amperometric signal (third trace from bottom) displayed peaks. The time derivative of the capacitance signal, which, like the amperometric signal, should be proportional to secretion rate, is displayed in the top trace (in femtofarads per second) for comparison with the latter.

METHODS. Whole-cell membrane current was recorded with a computer-controlled patch-clamp amplifier (EPC-9), which provides a routine for automatic compensation of cell capacitance. This routine can be repeated (capacitive tracking mode), with an analogue output signal proportional to capacitance. The latter signal was sampled together with the amperometric signal and with membrane voltage, averaged over 0.5 -s intervals, and stored on a computer for later analysis and plotting. The time derivative of the capacitance signal was calculated numerically, averaging over four neighbouring datapoints.

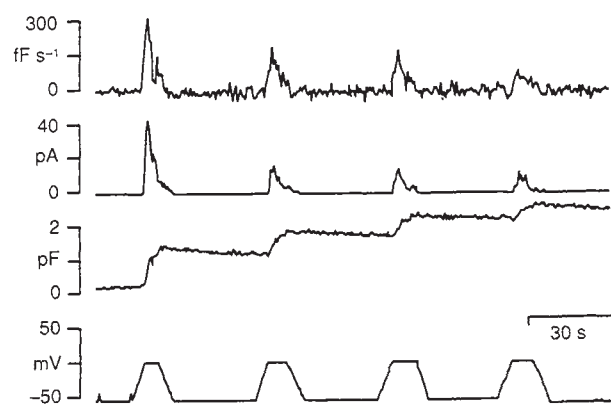
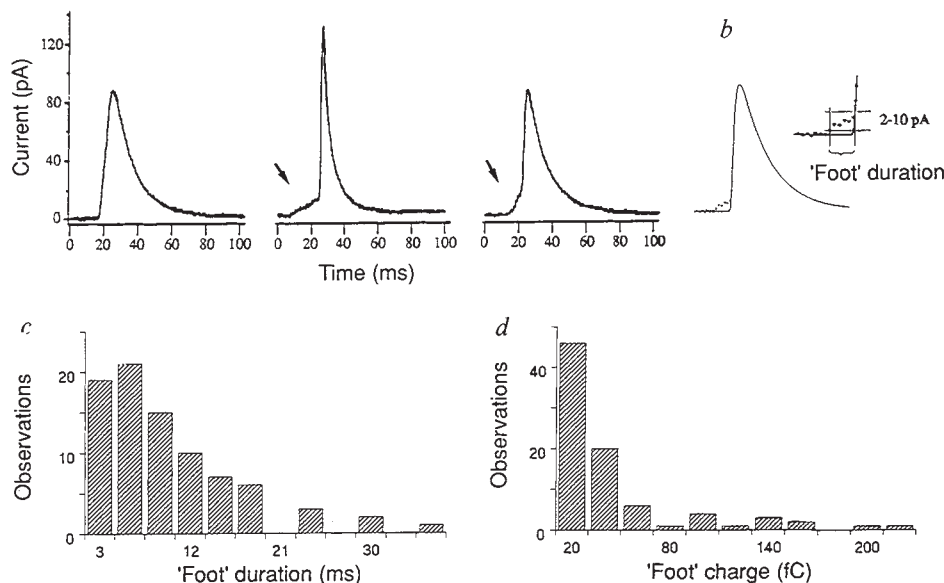


FIG. 3 Rising phase of amperometric current transients cannot be explained by instantaneous release of secretory vesicle contents. *a*, Single amperometric current transients at high resolution. Note that the fast rising phase in two out of the three examples is preceded by a small pedestal or 'foot' (arrows). Such 'foot' signals precede most of the transients with a fast rise time. The 'foot' signals were typically much smaller than the average signal. In one cell, 139 transients were counted, 51 of which were large (>20 pA). Of these large signals, 41 had a small (<20 pA) 'foot' starting within 20 ms of the main event, and in only two cases were there two large events within 20 ms of each other. If the 'foot' events followed the same amplitude distribution as the main events, one would expect about 12 to be large. *b*, Simulation of transient. The trace shown is the analytical solution for one-dimensional diffusion for the instantaneous release of 4.5 million molecules from the surface of an infinite reflecting plane, separated by distance x from a parallel infinite absorbing plane (as determined using the standard method of image planes^{26,27}). The flux J into the absorbing plane is given by

$$J = \sum_i (-1)^i \frac{M(x-2ix)}{2t\sqrt{\pi Dt}} \exp\left(\frac{-(x-2ix)^2}{4Dt}\right)$$

with $M = 4.5 \times 10^6$, $D = 3 \times 10^{-7}$ cm² s⁻¹, $x = 10^{-4}$ cm, and the sum extended from $i = -7$ to $i = 8$. Drawn superimposed over part of the analytical solution is a typical 'foot'. Inset, 'Foot' region shown at expanded scale, with markers denoting the parameters measured in our analysis procedure. We defined the beginning of the 'foot' as the time when the signal exceeded the peak-to-peak noise of a 5 -ms time segment (typically 1 – 2 pA) and the



end of the 'foot' as the time point when the signal exceeded a typical 'foot' amplitude (≈ 10 pA). When these criteria were applied to the analytical signal, a 'foot' duration of 0.7 ms was obtained. Thus, the inaccuracy of our 'foot duration' should be that order of magnitude. *c*, Histogram of duration of 'foot' signals. Data from three cells are pooled, where 84 large (>20 pA) and fast (rise time < 3 ms) signals were counted, 75 of those were preceded by a discernible 'foot'. The number of occurrences is plotted against the duration of the 'foot', as defined in *a*. The mean of 'foot' durations was 8.26 ms. Based on the latency distribution one would expect the mean interval to be 20 – 30 ms, if the 'foot' and the main event were independent. *d*, Histogram of 'foot' charges. The same 'foot' signals as those of *c* were integrated to calculate the charge involved. Mean charge: 34 fC or 1.05×10^5 molecules. The mean amplitude of the 'foot' signal was 7.17 ± 5.28 pA (mean $\pm$ s.d.).

explanation could be that the pedestal arises from the slow leakage of catecholamines through a so-called 'fusion pore' which is postulated to occur as an early step in exocytosis¹⁶. Fusion pores are believed to be gap-junction-like structures¹⁷ which form as a nucleus for fusion, and then, after some delay, dilate to complete the fusion process.

The observation of the long latency for exocytosis in chromaffin cells has profound implications for the underlying molecular mechanisms. At the neuromuscular junction and at the squid giant synapse fast kinetics of transmitter release have been explained either by a direct effect of voltage on the release process¹⁸ or alternatively by the fast buildup and collapse of microdomains of highly elevated calcium concentration^{19,20}. Such domains, in which the internal Ca^{2+} concentration can rise above 100 μM , are expected to occur in the immediate vicinity of open calcium channels. Neither of these mechanisms

seems to apply to the 'slow' secretion from neuroendocrine cells. The majority of secretory events occurs 5 – 100 ms after the end of a voltage pulse stimulus. Thus, membrane potential has been long restored to resting values, and Ca^{2+} -microdomains have collapsed (this occurs within microseconds after closing of channels) by the time the vesicles fuse. A theory explaining exocytosis in chromaffin cells must therefore assume either that calcium or membrane depolarization triggers a cascade of events that manifests itself only later, or else that exocytosis occurs predominantly at intermediate calcium concentration (5 – 30 μM), which prevail in a submembraneous shell for times of about 100 ms²¹.

Note added in proof: Since the submission of this paper, Wightman *et al.*²⁸ have published a paper that provides further evidence that the individual amperometric signals are due to single vesicle fusion events. □

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Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha 4\beta 1$ integrin

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EXPERIMENTAL autoimmune encephalomyelitis (EAE) is an inflammatory condition of the central nervous system with similarities to multiple sclerosis^{1,2}. In both diseases, circulating leukocytes penetrate the blood-brain barrier and damage myelin, resulting in impaired nerve conduction and paralysis³⁻⁵. We sought to identify the adhesion receptors that mediate the attachment of circulating leukocytes to inflamed brain endothelium in EAE, because this interaction is the first step in leukocyte entry into the central nervous system. Using an *in vitro* adhesion assay on tissue sections, we found that lymphocytes and monocytes bound selectively to inflamed EAE brain vessels. Binding was inhibited by antibodies against the integrin molecule $\alpha 4\beta 1$, but not by antibodies against numerous other adhesion receptors. When tested *in vivo*, anti- $\alpha 4$ integrin effectively prevented the accumulation of leukocytes in the central nervous system and the development of EAE. Thus, therapies designed to interfere with $\alpha 4\beta 1$ integrin may be useful in treating inflammatory diseases of the central nervous system, such as multiple sclerosis.

EAE was induced in Lewis rats by a single intraperitoneal injection of a CD4-positive T-cell clone specific for myelin basic protein⁶. These cells have been shown to localize in the central nervous system (CNS) within 4-12 h, where they initiate inflammation⁷. Endogenous monocytes and lymphocytes infiltrate inflamed vessels in the brain stem and spinal cord, leading to paralysis of the tail and hind limbs on days 4 and 5. The degree of leukocyte infiltration at the time of paralysis is shown in Fig. 1. Inflamed vessels were never found in brains of control animals.

TABLE 1 Antibodies against $\alpha 4\beta 1$ integrin inhibit U937, monocyte and lymphocyte binding to inflamed vessels in EAE brain sections

(a) Treatment of U937 cells		Relative binding to EAE vessels (% of control)	
Anti- β 1 integrin	AIIB2	8 $\pm$ 3	
Anti- α 3 integrin	A043	111 $\pm$ 5	
Anti- α 4 integrin	HP2/1 (inhibits FN and VCAM-1-binding)	3 $\pm$ 1	
Anti- α 4 integrin	P4G9 (selectively inhibits FN binding)	151 $\pm$ 7	
Anti- α 4 integrin	HP1/7 (selectively inhibits FN binding)	338 $\pm$ 41	
Anti- α 5 integrin	P1D6	104 $\pm$ 6	
Anti- α 6 integrin	GoH3	88 $\pm$ 11	
Anti- α 4, and - α 5	P4G9 and P1D6 (combined)	138 $\pm$ 8	
Anti- α 3, - α 4, and - α 6	A043, P1D6, and GoH3 (combined)	112 $\pm$ 3	
Anti-CD44	Hermes-3	107 $\pm$ 4	
Anti-L-selectin	TQ-1	96 $\pm$ 4	
Anti- β 2 integrin	P4H9	77 $\pm$ 1	
Anti- β 2 integrin	TS1/18	98 $\pm$ 5	
Anti- β 2 integrin	P4H9 (tested at 25 $^{\circ}$ C)	100 $\pm$ 10	
Anti- β 2 integrin	TS1/18 (tested at 25 $^{\circ}$ C)	114 $\pm$ 2	
Anti- α 4 integrin	HP2/1 (tested at 25 $^{\circ}$ C)	5 $\pm$ 1	
Anti-LFA-1	IOT16	123 $\pm$ 2	
Anti-Mac-1	LM2/1	107 $\pm$ 3	
(b) Treatment of freshly isolated lymphocytes or monocytes		Relative binding to EAE vessels (% of control)	
Antibody specificity	Clone	Cell type	
Anti- β 1 integrin	AIIB2	human lymphocytes	7 $\pm$ 2
Anti- α 4 integrin	HP2/1	human lymphocytes	0 $\pm$ 0
Anti- α 4 integrin	HP2/1	human monocytes	1 $\pm$ 1
Anti- α 4 integrin	HP2/1	rat lymphocytes	18 $\pm$ 7
Anti- α 4 integrin	R1-2	mouse lymphocytes	43 $\pm$ 2
Anti-CD2	OX-34	rat lymphocytes	100 $\pm$ 10
Anti-L-selectin	MEL-14	mouse lymphocytes	92 $\pm$ 4
Peyer's patch homing receptor	1B.2.6	rat lymphocytes	117 $\pm$ 12
Anti-LFA-1	OX-52	rat lymphocytes	87 $\pm$ 1
Anti-CD45	OX-1	rat lymphocytes	90 $\pm$ 3
Anti-Thy 1.1	OX-7	rat lymphocytes	087 $\pm$ 3
Anti-CD4	OX-35	rat lymphocytes	107 $\pm$ 5
Anti-monocyte/T cell surface	OX-44	rat lymphocytes	102 $\pm$ 8

a. Analysis of antibodies against cell adhesion molecules revealed that only reagents against $\alpha 4\beta 1$ integrin significantly affected binding of U937 cells to EAE vessels. Expression of $\beta 1$ integrins by U937 given as mean fluorescence channel number determined by FACS analysis: no primary antibody, 4; $\alpha 1$ (TS2/7), 3; $\alpha 2$ (G19), 5; $\alpha 3$ (A043), 25; $\alpha 4$ (HP2/1), 189; $\alpha 5$ (P1D6), 62; $\alpha 6$ (GoH3), 40.

b. The attachment of human peripheral blood lymphocytes and monocytes to EAE vessels was also inhibited by the anti-human $\alpha 4$ integrin antibody, HP2/1. This antibody crossreacts with rat lymphocytes and inhibits their binding to inflamed brain venules (although threefold higher concentrations of HP2/1 were required for maximal inhibition, and inhibition was not as complete as with human lymphocytes). An antibody against mouse $\alpha 4$ integrin (R1-2) was less effective but substantially inhibited the attachment of mouse lymphocytes to EAE vessels. Cells were treated with saturating concentrations (determined by FACS analysis) of the indicated antibodies on ice for 30 min before the *in vitro* section assay, as described in Fig. 2—which was usually done in the continued presence of the antibody. In several experiments, the cells were washed out of HP2/1 (anti- $\alpha 4$) and AIIB2 (anti- $\beta 1$) with no diminution of their inhibitory effect. Binding was quantified using an internal standard population of cells as previously described³⁰. Numbers indicated in the table represent the percentage of binding relative to the control with no antibody ($\pm$ s.e.m.), and is based on counting cells bound to at least 10 vessels in each of 3-6 independent sections. Antibody sources: HP2/1, HP1/7 have been described¹⁴; AIIB2, gift from C. H. Damsky, University of California, San Francisco; Hermes-3, gift from E. C. Butcher, Stanford University; 1B.2.6, gift from Y. H. Chin, University of Miami; A043, P4G9, P1D6 and P4H9, Telios Pharmaceuticals, San Diego; Gi9, GoH3 and 10T16, AMAC, Westbrook, Maine; OX-1, OX-7, OX-34, OX-35, OX-44 and OX-52, Bioproductions for Science, Indianapolis; TS2/7, T Cell Sciences, Cambridge, Massachusetts; TQ-1, Coulter Immunology, Hialeah, Florida; TS1/18, ATCC number HB 203; LM2/1, ATCC number HB 204; R1-2, ATCC number HB 227; and MEL-14, ATCC number HB 132.

Sections of day 5 EAE brain were tested for their ability to support leukocyte attachment in a modified Stamper-Woodruff *in vitro* binding assay⁸. Human monocytic cells of line U937 bound selectively to inflamed venules exposed in 10- μ m sections of EAE brain (Fig. 2), and occasionally to small arterioles (not shown), but never to vessels in sections of noninflamed brain. Binding was restricted to the lumen of the vessels, consistent with an endothelial interaction (Fig. 2). Comparable binding

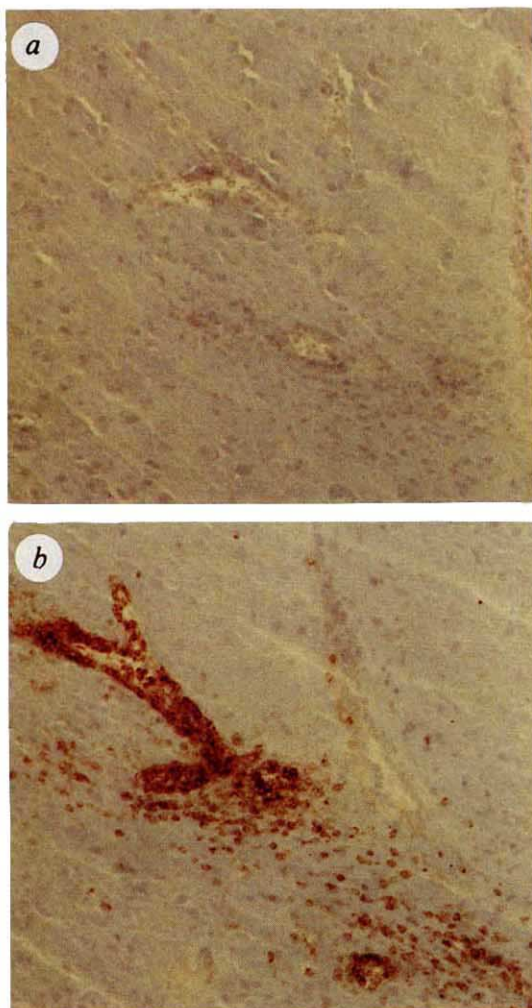


FIG. 1 Lymphocytes and monocytes infiltrate venules in the brainstem of Lewis rats during EAE. Immunostained frozen sections of brainstem taken from Lewis rats exhibiting tail and hind limb paralysis caused by EAE ($\times 70$ magnification). Sections were treated with: *a*, no primary antibody; *b*, monoclonal antibody OX-1, against CD45 which is expressed on all leukocytes; *c*, monoclonal antibody ED1, which recognizes circulating monocytes. Infiltrating leukocytes, comprised mostly of T cells (CD2⁺, not shown) and circulating monocytes (ED1⁺), form 'cuffs' around numerous venules in the brainstem, spinal cord and a few vessels in the cortex. ED1- and CD45-positive cells were always found in brain sections from animals exhibiting paralysis, and were never seen in brain sections from control animals.

METHODS. EAE was induced in Lewis rats by injecting 8×10^6 cells of a CD4⁺ T-cell clone specific for myelin basic protein (K-BP2). Production and maintenance of the T-cell clone was as previously described⁶. Animals exhibited tail or tail and hind limb paralysis on days 4 and 5, and brains were removed on day 6 for analysis. Immunohistochemistry was done on cryostat sections of brain tissue with Vectastain ABC (Vector Laboratories, Burlingame, California). The monoclonal antibodies, OX-1, OX-34 (against rat CD2), and ED1 were obtained from Bioproducts for Science.

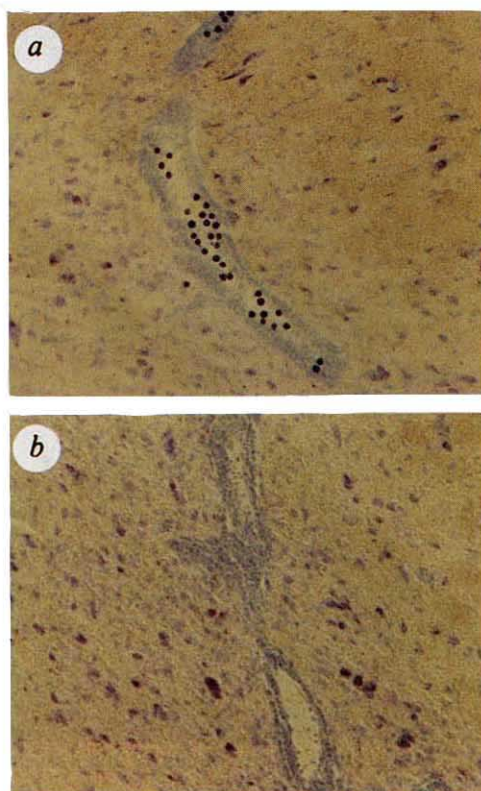


FIG. 2 *In vitro* binding of human U937 monocytic cells to inflamed vessels in rat EAE brain. *a*, ($\times 70$ magnification). Cryostat sections of EAE rat brain were exposed to U937 cells (ATCC number CRL 1593). Cells bound exclusively to inflamed venules and occasional arterioles. Attached cells are easily discernable because they stain more darkly than the sectioned tissue and because they are in a different focal plane. Several observations suggest that U937 bound to endothelium exposed in the profile of the inflamed vessel. (1) Binding was always to the lumen of the inflamed vessels; (2) under high power with differential focusing, U937 was consistently localized over endothelial cells; (3) U937 did not appear to interact with leukocytes surrounding the vessel, as there was no binding to the vessel where the lumen was out of the plane of section, nor to leukocytes that had migrated into the brain, away from the vessel. U937 also bound to the lumen of small arterioles in the EAE brain, which were never cuffed by endogenous leukocytes. *b*, In the same experiment, U937 cells were pretreated with anti- $\alpha 4$ integrin (HP2/1) and exposed to an adjacent section of EAE brain ($\times 70$ magnification). HP2/1 inhibited U937 binding to the inflamed venules by greater than 95% (Table 1).

METHODS. Adhesion was assayed using freshly cut, unfixed, 10 μ m sections of EAE or normal rat brain. Leukocytes, suspended in RPMI 1640 medium (with 10 mM HEPES and 5% FBS) at a final concentration of 10^7 cells ml^{-1} , were layered over each section, the slides were immediately placed on a metal tray supported by ice and gyrated at 60 r.p.m. for 30 min. Some assays were at 25 $^{\circ}\text{C}$. Details of the assay have been previously described³⁰. Human lymphocytes were isolated from EDTA-treated blood with Lymphoprep (Nycomed AS, Oslo), monocytes with Nycodenz (Nycomed AS), and neutrophils with Mono-Poly Resolving Medium (Flow Laboratories, Irvine, Scotland). Mouse and rat lymphocytes were isolated by crushing mesenteric and cervical lymph nodes and flushing with RPMI/HEPES/serum containing 40 $\mu\text{g ml}^{-1}$ DNase.

was obtained with human peripheral blood monocytes and lymphocytes, and with freshly isolated rat and mouse lymphocytes. Human peripheral blood neutrophils did not bind to EAE vessels.

Monoclonal antibodies against leukocyte adhesion receptors were examined for inhibitory activity in the *in vitro* section assay (Table 1). The attachment of U937 cells was almost completely inhibited by an antibody against human $\beta 1$ integrin. The $\beta 1$ integrins are a family of heterodimeric adhesion receptors, also known as VLA antigens^{9,10}. These molecules share a common β chain ($\beta 1$) in conjunction with six unique α chains. U937 expresses $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ of the $\beta 1$ integrins (Table 1). Function-blocking, α chain-specific antibodies against these molecules were tested for inhibition of U937 binding to EAE vessels; anti- $\alpha 4$ (clone HP2/1) produced greater than 95% inhibition (Fig. 2 and Table 1a), whereas anti- $\alpha 3$, anti- $\alpha 5$ and anti- $\alpha 6$ were without significant effect, even when administered in combination (Table 1a). Thus, $\alpha 4\beta 1$ integrin is required for U937 adhesion to EAE vessels. These results were confirmed with freshly isolated rat and mouse lymphocytes and with lymphocytes and monocytes from human peripheral blood (Table 1b).

$\alpha 4\beta 1$ integrin has two distinguishable binding domains, one that mediates adhesion to fibronectin (FN) and another that binds the endothelial ligand VCAM-1 (refs 11, 12; INCAM-110, ref. 13). HP2/1, which prevented U937 binding to EAE vessels, inhibits both functional activities of $\alpha 4$ integrin¹⁴. Surprisingly, two antibodies that selectively inhibit the FN-binding activity of $\alpha 4$ integrin (P4G9 and HP1/7), enhanced U937 attachment to the EAE vessels. P4G9 also enhanced vessel binding when combined with anti- $\alpha 5$ integrin (Table 1a), whereas this combi-

nation of antibodies inhibited U937 binding to FN by over 90% (not shown). These results suggest that the FN-binding activity of $\alpha 4$ integrin is not directly involved in U937 adhesion to EAE vessels *in vitro*. Whether the known endothelial ligand for $\alpha 4\beta 1$ integrin, VCAM-1, is expressed on the EAE vessels awaits production of appropriate reagents, but VCAM-1 has been detected on inflamed venules in mouse brain (B. Engelhardt and E. C. Butcher, personal communication) and at other sites of inflammation *in vivo*^{15,16}.

Antibodies against many other leukocyte adhesion receptors were without effect on U937 or lymphocyte binding to EAE vessels (Table 1). Notable among these were anti-L-selectin (MEL-14), anti-CD 44 (Hermes-3), and 1B.2.6, which have been shown to inhibit lymphocyte attachment to venules in sections of lymph node (MEL-14, ref. 17) or Peyer's patches (CD-44, refs 18, 19, and 1B.2.6, ref. 20). Anti- $\beta 2$ integrin had little or no effect at either 4 °C or 25 °C (the activity of the $\beta 2$ integrins has been reported to be temperature dependent), whereas HP2/1 produced almost complete inhibition at either temperature. These results suggest that $\alpha 4\beta 1$ integrin has a prominent role in leukocyte adhesion to the inflamed brain venules. Other adhesion molecules are likely to work in conjunction with $\alpha 4\beta 1$ integrin for effective leukocyte attachment and migration across the vessel wall *in vivo*. For example, the $\beta 2$ integrins strengthen leukocyte adhesion after initial binding and have been implicated in subsequent leukocyte transmigration²². ICAM-1, a ligand for the $\beta 2$ integrins²³, has been observed on inflamed venules during EAE²⁴⁻²⁶.

As the anti- $\alpha 4$ integrin antibody, HP2/1, blocked lymphocyte and monocyte binding to EAE vessels *in vitro*, the effect of this antibody on the progression of EAE was tested *in vivo*

a Experiment 1			
Treatment	Number of animals with paralysis		
	Day 5	Day 6	Day 7
No antibody	4/6	5/6	4/6
HP2/1 (1.2 mg)	0/6	0/6	0/6
MOPC (1.2 mg)	6/6	6/6	5/6
Experiment 2			
Treatment	Number of animals with paralysis		
	Day 4	Day 5	Day 6
No antibody	5/5	5/5	5/5
HP2/1 (1.0 mg)	0/4	2/4	2/4
OX-1 (1.0 mg)	5/5	5/5	5/5
OX-7 (1.0 mg)	5/5	5/5	5/5
Experiment 3			
Treatment	Number of animals with paralysis		
	Day 4	Day 5	Day 6
No antibody	6/6	6/6	6/6
HP2/1 (0.4 mg)	1/5	2/5	2/5
HP2/1 (0.8 mg)	1/5	2/5	1/5
HP2/1 (1.6 mg)	0/6	0/6	2/6

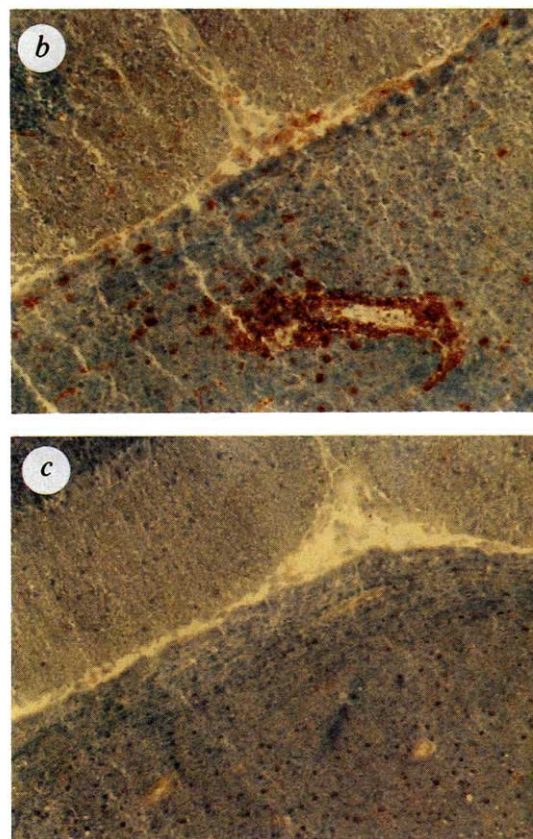


FIG. 3 *In vivo* administration of anti- $\alpha 4$ integrin prevents accumulation of leukocytes in the CNS during EAE and prevents paralysis. **a** In each experiment, the T-cell clone was administered on day 0. On day 2, animals received an intraperitoneal injection of PBS, the indicated amount of purified anti- $\alpha 4$ integrin, or the indicated amount of purified control antibody. All antibodies were mouse IgG₁. Disease was defined by complete tail or tail and hind limb paralysis. In animals that developed disease, paralysis began on day 4 or 5, peaked on day 5 or 6, and steadily diminished thereafter. Two additional experiments gave comparable results. Circulating levels and differential counts of white blood cells were indistinguishable between HP2/1-treated and PBS control animals when measured on days 3, 4 and 7 (day 3 is one day after antibody administration and one day before the earliest onset of paralysis). Bulk quantities of HP2/1 were purchased from AMAC; MOPC from Sigma; OX-1 and OX-7 from Bioproducts for Science. **b**, **c**, Brains were removed from several diseased EAE and healthy anti- $\alpha 4$ integrin-treated EAE rats from experiments 2 and 3 on day 6 ($\times 70$ magnification). The micrographs show sections of comparable regions of the

brainstem and cerebellum, immunostained with anti-CD45 (OX-1) to illustrate the degree of leukocyte infiltration. There was extensive infiltration in brains from diseased EAE animals (**b**), whereas leukocytes could not be detected in the EAE rats treated with anti- $\alpha 4$ integrin (**c**).

(Fig. 3a). Two days after administering the inflammation-inducing T-cell clone, animals were given a single intraperitoneal injection of HP2/1 or of control antibodies. Anti- $\alpha 4$ integrin completely prevented the development of paralysis in 75% of the treated animals; in those that developed disease, paralysis was delayed and its severity was reduced (Fig. 3a). Isotype-matched control antibodies that bind to the surface of rat monocytes and T-cells at higher amounts than HP2/1 did not affect the onset or severity of disease (Fig. 3a). The protective effect of HP2/1 was dose dependent. Nearly all animals were protected by higher concentrations of antibody (Fig. 3a). Immunohistochemically, brainstems from EAE animals injected with control antibodies revealed extensive leukocyte infiltration (Fig. 3b), whereas infiltration was not detected in brains from EAE animals treated with anti- $\alpha 4$ integrin.

Several mechanisms might explain the strong protective effect of anti- $\alpha 4$ integrin against infiltration of the CNS by host leukocytes and the progression of EAE. First, results with the *in vitro* section assay indicate that anti- $\alpha 4$ integrin inhibits the adhesive interactions of lymphocytes and monocytes with inflamed brain vessels, and thus may prevent circulating leukocytes from entering the CNS. Second, although the data suggest that the FN-binding site of $\alpha 4 \beta 1$ integrin is not involved in leukocyte adhesion to inflamed vessels, FN interactions may be important for leukocyte migration across the vessel wall²⁷. If so, blocking this interaction with the antibody HP2/1 could have contributed to the *in vivo* effects. Third, $\alpha 4 \beta 1$ integrin can augment antigen-specific activation of T cells^{28,29}. As EAE was initiated by injection of an antigen-specific T-cell clone, it is possible that antibody treatment inhibited necessary signals delivered to the clone through $\alpha 4 \beta 1$. This is unlikely because anti- $\alpha 4$ integrin was not administered until 2 days after injection of the T-cell clone, by which time the T cells would have already entered the CNS and initiated disease. Thus, we favour the hypothesis that antibody treatment blocked the entry of host lymphocytes and monocytes that would have normally infiltrated the brain in response to inflammation initiated by the T-cell clone. Experiments with control antibodies rule out the trivial explanation that any antibody reactive with circulating lymphocytes and monocytes would affect EAE (Fig. 3). Furthermore, absolute levels and relative counts of lymphocytes and monocytes were not affected by the antibody at any time during the course of disease (Fig. 3), indicating that the antibody did not induce leukocyte depletion.

Previous work on $\alpha 4 \beta 1$ -dependent cell adhesion has mainly involved studies with endothelium that has been grown and stimulated in culture. The *in vitro* section assay described here extends those observations by showing that $\alpha 4 \beta 1$ integrin is crucial for the adhesion of leukocytes to vessels that have been activated *in vivo*. Furthermore, *in vivo* administration of anti- $\alpha 4$ integrin prevented paralysis associated with the pathogenic inflammation of EAE. Therapy based on inhibiting $\alpha 4 \beta 1$ integrin, or the ligand for this receptor on brain endothelium, may prove effective in treating inflammatory diseases of the CNS. □

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Linkage of regression and malignant conversion of rabbit viral papillomas to MHC class II genes

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HUMAN papillomaviruses associated with cutaneous¹ and anogenital² cancers induce intraepithelial precursor lesions which may regress spontaneously or progress into invasive carcinomas^{3,4}. Cell-mediated immune responses are probably involved in regression of precancerous lesions^{1,5–8} and the polymorphism of the genes responsible may thus have a key role in the variability of the host response. Skin warts and cancers induced in rabbits by Shope papillomavirus^{9–12} provide a model to test this hypothesis. We analysed a restriction-fragment-length polymorphism of major histocompatibility complex class I and class II genes^{13,14} and T-cell receptor β -chain genes¹⁵ in infected domestic rabbits. We found a strong linkage between wart regression and a *DR α* *EcoRI* fragment, and an increased relative risk of malignant transformation associated with a *DQ α* *PvuII* fragment. This indicates a genetic control of wart evolution, involving genes in the class II region of the major histocompatibility complex.

Seventy-seven outbred New Zealand rabbits infected with the Shope cottontail rabbit papillomavirus (CRPV) developed skin warts that underwent early rapid regression in 42 animals in 1 to 6 weeks. A late progressive regression was observed in 12 rabbits after 8 to 14 weeks. Eleven of the 23 persistor rabbits developed invasive carcinomas (Table 1). We found significant associations between the evolution of warts and a restriction-fragment-length polymorphism (RFLP) of the rabbit major histocompatibility complex (MHC) class II *DR α* and *DQ α* genes. Four *EcoRI* fragments were identified, defining *DR α* RFLP alleles A to D (Fig. 1a), and seven *PvuII* fragments, defining *DQ α* RFLP alleles A to G (Fig. 1b). Strikingly, 25 out of 26 rabbits bearing *DR α B*, whether homozygous or heterozygous, showed early regression (Table 1), a significantly higher proportion than among rabbits with *DR α A* (13 out of 21), *C* (12 out of 37) or *D* (23 out of 48) ($P < 0.01$; Z test¹⁶). On testing the relative predispositional effect (RPE)¹⁷ of each *DR α* allele in wart evolution, the most significant deviation was the increased frequency of *DR α B* in early regressors (Table 2a). The significance of the decreased frequency observed for the *DR α C* allele was not confirmed when tested after excluding the *DR α B*

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TABLE 1 Distribution of rabbit *DRα* and *DQα* RFLP alleles as related to wart evolution

<i>DRα</i>	<i>DQα</i>	Number of regressors		Number of persistors	
		Early	Late	No cancer	Cancer
AA	AE	1	-	-	-
AB	AE	1	-	-	-
AB	EE	3	-	-	-
AC	AG	-	-	1	-
AC	BG	-	-	-	1
AC	CE	-	-	1	-
AC	EE	2	-	-	-
AD	BB	-	-	-	1
AD	BE	6	-	1	2
AD	CE	-	-	1	-
BB	EE	6	-	-	-
BB	EG	1	-	-	-
BC	CE	-	1	-	-
BC	EE	2	-	-	-
BC	EG	3	-	-	-
BD	BB	3	-	-	-
BD	BE	3	-	-	-
BD	CE	2	-	-	-
BD	EG	1	-	-	-
CC	BC	-	-	-	1
CC	BE	-	1	-	-
CC	BG	-	-	-	1
CC	CC	-	-	1	-
CC	CD	-	-	-	1
CC	GG	-	-	-	1
CD	AB	1	-	-	-
CD	BB	1	2	2	-
CD	BC	-	1	-	-
CD	BE	-	1	-	-
CD	BG	2	2	1	3
CD	CC	-	-	1	-
CD	CE	1	1	-	-
CD	CG	-	1	-	-
DD	BB	1	1	-	-
DD	BC	-	1	-	-
DD	BD	-	-	1	-
DD	BE	-	-	1	-
DD	BF	1	-	-	-
DD	BG	1	-	1	-
Totals		42	12	12	11

DRα and *DQα* RFLP alleles correspond to the four *EcoRI* and seven *PvuII* fragments of rabbit genomic DNA detected with *DRα* and *DQα* probes, respectively (see legend to Fig. 1). The number of rabbits is indicated for each genotype, as related to the evolution of skin warts induced by the CRPV. CRPV particles were purified from cottontail rabbit warts²⁶, suspended in phosphate-buffered glycerol (1:1, v/v) and applied onto a 10 cm² area of shaved skin abraded with sand paper, on each flank of 77 New Zealand White rabbits (strain HY2000; CEGAV, Passais la Conception, France). Each rabbit was inoculated with two different doses corresponding to about 5 and 50 times the minimal dose resulting in wart outgrowth. Warts developed 3–5 weeks after infection, the lower dose yielding 5–30 warts and the higher dose usually confluent warts. Complete regression occurred 1–6 weeks after wart outgrowth (early regressors) or after 8–14 weeks (late regressors). Persistor rabbits kept their warts for over 16 weeks and were followed up for 1 year for the development of carcinomas. Two died accidentally without cancer after 6 and 9 months. Four rabbits lost their warts between 18 and 49 weeks as a consequence of dead tissue shedding off rather than of an immune reaction¹⁰.

allele (Table 2a). For *DQα* alleles, rabbits with *DQαE* showed a significantly higher rate of early regression (32 out of 42) than rabbits without *DQαE* (10 out of 35) or with the most frequent *DQαB* allele (19 out of 44) ($P < 0.01$; Z test¹⁶) and all 13 homozygous *DQαE* rabbits were early regressors. Using the RPE method¹⁷, the increased frequency of *DQαE* was the only significant deviation in early regressors (Table 2a). The *DQαE* allele is present in 23 of the 26 regressor rabbits with *DRαB*, indicating a linkage disequilibrium between the two alleles found associated with early regression. Another interesting

finding was the significantly higher incidence ($P < 0.05$) of cancers among rabbits with *DQαG* allele (6 out of 20 versus 5 out of 57) (Table 1). By contrast, no significant difference was found for *DQαB*, the most frequent *DQα* allele in cancer-bearing animals (9 out of 44 versus 2 out of 33) (Table 1). *DQαG* was the allele contributing most to the significant deviation detected by the RPE method (Table 2b). On removal of *DQαG*, the overall χ^2 was no longer significant (Table 2b). The relative risk¹⁶ of developing a cancer associated with *DQαG* was evaluated to 4.45 with 95% confidence limits of 1.15–17.23 (Table 2).

No association was found between wart evolution and the RFLP of either MHC class I genes, detected with probes recog-

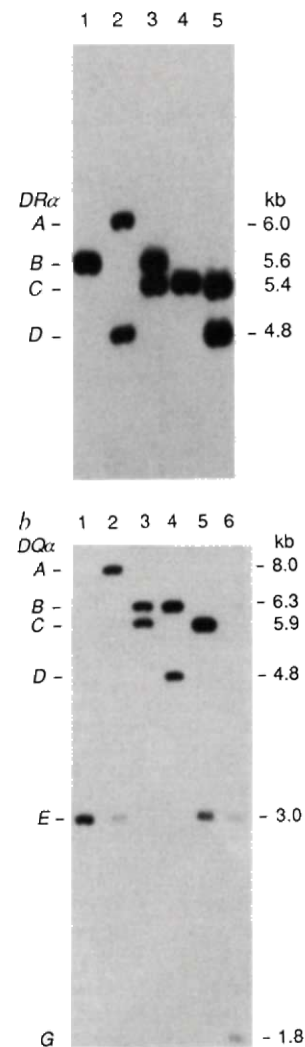


FIG. 1 RFLP of rabbit *DRα* and *DQα* genes. Genomic DNA was digested with *EcoRI* (a) or *PvuII* (b) endonucleases and analysed by Southern blot hybridization using probes specific for rabbit *DRα* (a) or *DQα* (b) genes. Fragments defining alleles are identified by letters on the left of the figures and by their sizes expressed in kilobases (kb) on the right. Fragment *DRαF* (2.4 kb) found only once is not shown. Homozygous rabbits show single-band patterns (a, lanes 1, 4; b, lane 1) and heterozygotes 2-band patterns (a, lanes 2, 3, 5; b, lanes 2 to 6), in agreement with previous reports^{13,14}.

METHODS. Genomic DNA was prepared from peripheral white blood cells¹³. DNA preparations (5 µg) were digested with *EcoRI* (a) or *PvuII* (b) endonucleases at 37 °C for 16 h, in conditions indicated by the supplier (Amersham). DNA fragments were separated by electrophoresis in 1% agarose gels and transferred to nitrocellulose membranes (Schleicher Schuell, BA85). The *DRα* probe was a 2-kb fragment containing exon 3 and extending to the 3' untranslated region¹³. The *DQα* probe was a 2.2-kb fragment extending from exon 2 to the 3' untranslated region¹³. ³²P-labelled probes (specific activity >10⁹ c.p.m./µg⁻¹) were obtained by random priming (Amersham). Membranes were hybridized overnight at 42 °C in 50% formamide/5 × SSC, washed at 65 °C in 0.1 × SSC/0.1% SDS and autoradiographed.

TABLE 2 Relative predispositional effects of *DRα* and *DQα* RFLP alleles

(a) Early regressor rabbits

RFLP alleles	Round 1 of comparison				Round 2: <i>DRαB</i> or <i>DQαE</i> removed		
	Number observed	Number expected	χ^2	P value	Number expected	χ^2	P value
<i>DRα</i>							
A	14	12	0.33		9.46	2.18	
B	32	8	<u>10.88</u>	<0.05	—	—	
C	12	23.46	<u>5.59</u>	<0.05	18.48	2.27	
D	26	30.54	0.67		24.06	0.16	
Totals	84	84	<u>17.47</u>	<0.0006	52	4.61	n.s.
<i>DQα</i>							
A	3	2.18			1.58		
B	24	30	1.20*		21.66	0.25	
C	3	8.72			6.30		
D	0	1.10			0.78		
E	45	30	<u>7.50*</u>	<0.05	—	—	
F	1	0.54			0.40		
G	8	11.46	<u>1.04*</u>		8.28	0.01	
Totals	84	84	<u>9.74</u>	<0.007	39	0.26	n.s.

(b) Persistor rabbits with cancer

RFLP alleles	Round 1 of comparison				Round 2: <i>DQαG</i> removed		
	Number observed	Number expected	χ^2	P value	Number expected	χ^2	P value
<i>DQα</i>							
A	0	0.57			0.45		
B	10	7.86	0.29*		6.20	1.69	
C	2	2.29			1.81		
D	1	0.28			0.23		
E	2	7.86	2.61*		6.20		
F	0	0.14			0.11	1.27	
G	7	3.0	<u>4.74*</u>		—	—	
Totals	22	22	<u>7.64</u>	<0.02	15	2.96	n.s.

The relative effects of each allele in wart evolution was examined by testing the fit of allele frequencies to expectations using the RPE¹⁷ method. In the absence of reference values for domestic rabbits, expected allele frequencies were calculated from the overall frequencies observed in our series of rabbits (0.143, 0.214, 0.279, 0.364 for *DRαA,B,C,D*, and 0.026, 0.357, 0.104, 0.013, 0.357, 0.007, 0.136 for *DQαA,B,C,D,E,F,G*). The method sequentially tests allele frequencies to determine their predispositional, protective or neutral effects relative to each other. The statistical comparison of overall frequencies was done, using a χ^2 test¹⁶. When the overall χ^2 was statistically significant (underlined, P value), the allele with the largest contribution (underlined) was removed and the test repeated using expected frequencies normalized accordingly, until the overall deviation was nonsignificant (n.s.). Significant deviation in each allele frequency was determined by a Z test¹⁶ and the relative risk¹⁶ and its confidence limits by the 4F BMDP program (BMDP Software, Los Angeles). Significant deviations in the overall frequency distribution of *DRα* and *DQα* alleles were detected only in early regressor rabbits (a) and in persistor rabbits with cancer (b).

* Because of their small number, *DQαA*, *DQαC* and *D*, and *DQαF* were combined to *DQαB*, *DQαE* and *DQαG*, respectively.

nizing about half of the genes¹³ (eleven *Bam*HI fragments) and an additional single copy gene¹³ (three *Bgl*II fragments), or the constant region of the T-cell receptor β genes¹³ (five *Bam*HI fragments).

In the multistage carcinogenesis associated with papillomaviruses, host immune responses control the expression of the viral oncogenic potential by conditioning regression or persistence of precancerous lesions. Most likely targets are the viral E6 and E7 oncoproteins^{8,18} which are expressed through all stages of tumour progression in rabbits^{19,20} and humans². Infiltrates of lymphocytes and macrophages in regressing warts^{10,21} suggest a role for cytotoxic T lymphocytes and for cytokines in reducing cell proliferation¹². We found that the capacity of rabbits to reject warts is strongly associated with two linked alleles of genes encoding class II DR and DQ antigens. MHC class II antigens, which display similarities in rabbit and in man^{13,14} consist of an α chain and a β chain, whose polymorphic N-terminal $\alpha 1$ and $\beta 1$ domains are involved in the binding of foreign antigenic peptides and their presentation to T lymphocytes²². Whether the *DRα* allele always found associated with regression is directly involved or is tightly linked to a gene responsible for regression has to be determined. Our preliminary sequence data do not support a correlation of the RFLP of the *DRα* gene with variations in the amino-acid

sequence of the $\alpha 1$ domain (unpublished observation). Whether wart regression depends on the polymorphism of the closely linked (about 6 kilobases away) *DRβ* genes¹⁴ is under study.

Our data support the existence of a *DQα*-linked gene involved in cancer development in rabbits. This is especially interesting in the light of the association recently reported between the HLA-DQw3 antigen and susceptibility to development of cervical cancer²³, known to be aetiologically associated with human papillomaviruses². A candidate in the MHC could be the gene encoding tumour necrosis factor- α (ref. 24), a cytokine with potent antitumour activity and an important role in inflammatory processes and immunological responses in the skin²⁵.

Our studies suggest some immunogenetic controls of the evolution of precancerous papillomavirus-associated lesions towards regression or malignant transformation. The rabbit model should prove useful in disclosing some of the underlying mechanisms. □

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Product of vav proto-oncogene defines a new class of tyrosine protein kinase substrates

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SEVERAL proteins implicated in the regulation of cellular responses to mitogenic stimuli contain a common non-catalytic domain, SH2 (for *src*-homologous domain 2), that mediates their interaction with activated tyrosine protein kinases. Here we report that p95^{vav}, a proto-oncogene product specifically expressed in cells of the haematopoietic system, contains an SH2 domain and is a substrate for tyrosine protein kinases. Exposure of quiescent NIH3T3 cells ectopically expressing p95^{vav} to either epidermal or platelet-derived growth factors induces the rapid phosphoryla-

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tion of this protein on tyrosine residues. Activation of the receptors for these growth factors by their cognate ligand results in their association with $p95^{vav}$, a process mediated by its SH2 domain. In T cells, co-activation of the T-cell receptor and the accessory CD4 cell-surface protein also results in the phosphorylation of the endogenous $p95^{vav}$ protein in tyrosine residues. Phosphorylation of $p95^{vav}$ is rapid, transient and precedes the appearance of most other phosphotyrosine-containing proteins. In addition to the SH2 domain, $p95^{vav}$ contains structural motifs not found in other tyrosine kinase substrates. One such motif is a helix-loop-helix/leucine zipper-like domain which shares some sequence similarity with these motifs in the Myc and Max proteins. Deletion of the helix-loop-helix-like motif causes oncogenic activation of $p95^{vav}$. These results indicate that $p95^{vav}$ is a new type of signal transduction molecule and suggest a possible role for this protein in the transduction of tyrosine phosphorylation signalling into transcriptional events.

The *vav* gene was first identified by virtue of its oncogenic activation during the course of gene transfer assays¹. Its gene product, $p95^{vav}$ or Vav (relative molecular mass (M_r) 95,000) contains an array of structural motifs^{2,3}, including an SH2 domain⁴ (Fig. 1a), which could indicate that this protein serves as a substrate for tyrosine protein kinases. NIH3T3 cells transfected with a expression plasmid encoding the mouse *vav* proto-oncogene (B36-212 cells) were immunoprecipitated with polyclonal antibodies prepared against a synthetic peptide corresponding to residues 576–589 of $p95^{vav}$. The resulting immunoprecipitates were submitted to western blot analysis using either anti-Vav or anti-phosphotyrosine antibodies. As shown in Fig. 1b, both of these antibodies recognized a protein

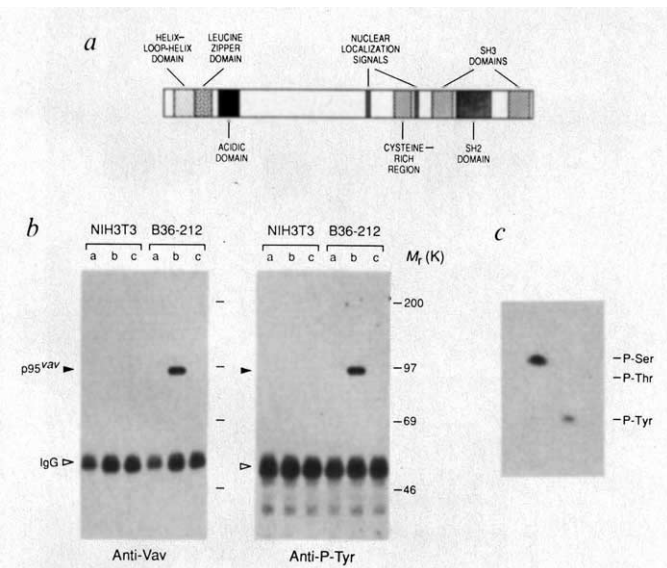


FIG. 1 Phosphorylation of $p95^{vav}$ on tyrosine residues. *a*, Structural motifs identified in $p95^{vav}$. *b*, Immunoblot analysis of immunoprecipitates from cellular extracts of parental NIH3T3 cells and NIH3T3 cells expressing the mouse *vav* proto-oncogene (B36-212 cells)². Cells were lysed in denaturing RIPA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet-P40, 1% sodium deoxycholate, 1% aprotinin, 250 μ M PMSF, 1 mM NaF and 100 μ M sodium orthovanadate) and immunoprecipitated with either (lane a) pre-immune sera or (lanes b, c) immune sera raised against a synthetic peptide corresponding to residues 576–589 of $p95^{vav}$ (ref. 2) in the (lane b) absence or (lane c) presence of competing peptide. Immunoprecipitates were fractionated by SDS-PAGE, transferred to nitrocellulose filters and incubated with either polyclonal anti-Vav antibodies or with monoclonal antibodies against phosphotyrosine residues (anti-P-Tyr; Upstate Biotechnology)¹⁹. Immunoreactive bands were visualized using ¹²⁵I-labelled protein A followed by autoradiography. The migration of $p95^{vav}$ and the IgG heavy chain is indicated by solid and open arrowheads, respectively. *c*, Phosphoamino-acid analysis²⁰ of $p95^{vav}$ immunoprecipitated from [³²P]-orthophosphate-labelled B36-212 cells. The migration of phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) is indicated.

of $M_r \sim 95,000$ (95 K) in the NIH3T3 cells transfected with the *vav* proto-oncogene, but not in the parental NIH3T3 cells. This protein was identified as $p95^{vav}$ because it could not be detected in those immunoprecipitates generated either in the presence of the immunizing peptide or with preimmune sera (Fig. 1b). To confirm the existence of phosphotyrosine residues in $p95^{vav}$, we isolated this protein from [³²P]orthophosphate-labelled B36-212 cells and analysed its phosphoamino-acid content. As illustrated in Fig. 1c, $p95^{vav}$ contains phosphoserine, phosphotyrosine and traces of phosphothreonine.

We next investigated whether $p95^{vav}$ became phosphorylated on tyrosine residues as a response to epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), two well characterized mitogens that mediate their effects through activation of the tyrosine kinases of their respective receptors. As shown in Fig. 2, the phosphotyrosine content of $p95^{vav}$ was barely detectable in quiescent B36-212 cells. But when these cells were treated with EGF or PDGF, we observed a significant increase in the tyrosine phosphorylation of $p95^{vav}$. The rate of $p95^{vav}$ phosphorylation in the EGF-treated cells paralleled that of the EGF receptor itself, reaching maximum levels within one minute after the addition of EGF (Fig. 2a). In agreement with previous studies, EGF-induced phosphorylation of the EGF receptor decreases rapidly (within 10 min) after EGF exposure owing to receptor internalization. In contrast, the levels of tyrosine phosphorylation in $p95^{vav}$ remained constant for at least 2 h (Fig. 2a, upper panel). Similar results were obtained when quiescent B36-212 cells were treated with PDGF (Fig. 2b). These results raised the possibility that $p95^{vav}$ might be directly phosphorylated by the activated receptors.

To verify this hypothesis, we tested whether $p95^{vav}$ was associated with these receptors. EGF receptor immunoprecipitates from either untreated or EGF-treated B36-212 cells were analysed by western blotting using anti-Vav antibodies. No significant amount of $p95^{vav}$ could be detected in immunoprecipitates derived from quiescent B36-212 cells (Fig. 3a). But when these cells were stimulated with EGF, $p95^{vav}$ was found to co-immunoprecipitate with the EGF receptor (Fig. 3a). Similar results were obtained when B36-212 cells were incubated with antibodies raised against the PDGF receptor (Fig. 3b). These results indicate that $p95^{vav}$ can form a stable complex with the

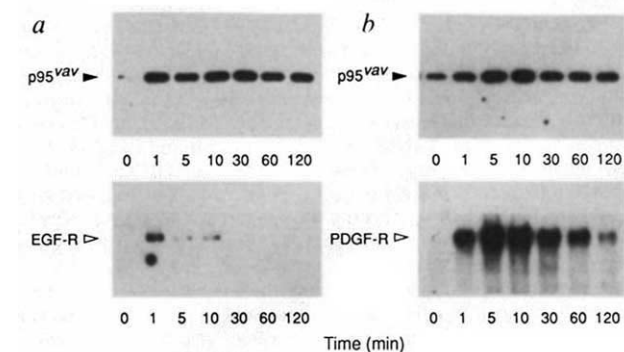


FIG. 2 Time course of $p95^{vav}$ tyrosine phosphorylation in EGF- and PDGF-stimulated B36-212 cells. B36-212 cells were made quiescent by culturing 95% confluent dishes for 48 h in DMEM containing 0.5% calf serum plus 5 μ g m^{-1} insulin and transferrin. Quiescent cells were subsequently treated with either EGF (1 μ g ml^{-1} ; Collaborative Res.) or PDGF (0.1 μ g ml^{-1} of human recombinant PDGF-AB; Upstate Biotechnology) for the indicated times. After stimulation, cells were lysed in non-denaturing (without SDS) RIPA buffer and immunoprecipitated with the corresponding antibodies. *a*, Anti-P-Tyr immunoblot analysis of $p95^{vav}$ (upper panel) and EGF receptor (lower panel) immunoprecipitates. *b*, Anti-P-Tyr immunoblot analysis of $p95^{vav}$ (upper panel) and PDGF receptor (lower panel) immunoprecipitates. The migration of $p95^{vav}$ (closed arrowheads) and the EGF and PDGF receptors (open arrowheads) is indicated.

EGF and PDGF receptors and that this association requires their prior activation by their cognate ligands.

To determine whether the binding of p95^{vav} to the EGF and PDGF receptors is mediated by its SH2 domain, we expressed those sequences encoding the Vav SH2 domain in *Escherichia coli* using the pMALc expression vector system. As shown in Fig. 3c, the maltose-binding protein (MBP)-Vav SH2 fusion protein, but not a control MBP- β -galactosidase (β -gal) fusion polypeptide, specifically complexed with a 180K phosphotyrosine-containing protein in lysates of EGF-stimulated F19-91 cells, an NIH3T3 cell line expressing the human EGF receptor. Immunoblotting of duplicate samples with antibodies against the EGF receptor confirmed that this protein was indeed the EGF receptor (Fig. 3c). Interestingly, the MBP-Vav SH2 fusion protein also complexed with a number of other tyrosine-phosphorylated proteins in EGF-stimulated F19-91 cells (Fig. 3c).

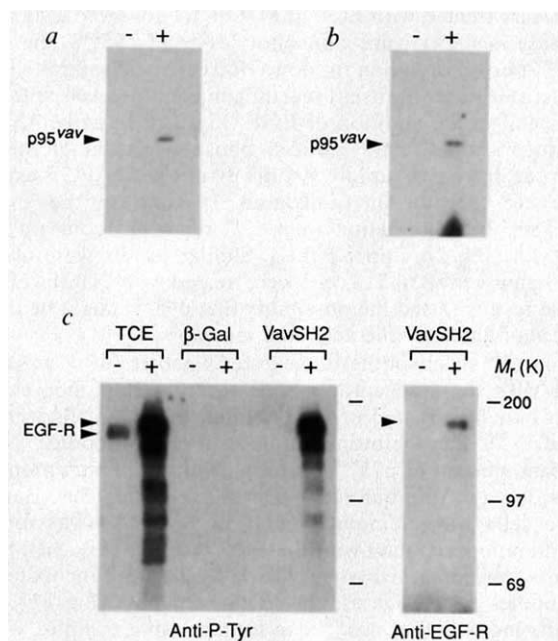


FIG. 3 p95^{vav} associates with the activated EGF and PDGF receptors through its SH2 domain. *a*, Anti-Vav antiserum immunoblot analysis of EGF receptor immunoprecipitates obtained from B36-212 cells either unstimulated (-) or stimulated (+) with EGF for 1 min. *b*, Same analysis of PDGF receptor immunoprecipitates obtained from B36-212 cells either unstimulated (-) or stimulated (+) with PDGF for 10 min. The arrows denote the migration of p95^{vav}. *c*, Western blot analysis using anti-P-Tyr (left panel) or EGF receptor (right panel) antibodies of cellular extracts derived from F19-91 cells (see below) either unstimulated (-) or stimulated (+) with EGF for 1 min. TCE, untreated total F19-91 cellular extracts; β -Gal, F19-91 extracts incubated with the control MBP- β -Gal fusion protein, and Vav SH2, F19-91 extracts incubated with the MBP-Vav SH2 fusion protein. Arrows indicate the migration of the EGF receptor.

METHODS. Immunoblotting is described in the legend to Fig. 2. Sequences encoding the murine Vav SH2 domain were amplified by polymerase chain reaction using the pJC11 plasmid as template² and subcloned into the pMALc expression vector (NE Biolabs). Cultures of *E. coli* DH α with pMALc expression plasmids containing the maltose-binding protein (MBP) fused to either the LacZ gene (β -Gal) or the Vav SH2 domain were processed as suggested by the manufacturer. The MBP fusion proteins were purified by affinity chromatography using an amylose matrix (NE Biolabs) and eluted in RIPA buffer containing 10 mM maltose. After elution, fusion proteins were immobilized in protein A-Sepharose beads precoated with anti-MBP antibodies (NE Biolabs). F19-91 cells overexpressing the human EGF receptor were generated by transfection of NIH3T3 cells with pHERN3B, an expression plasmid encoding the human EGF receptor (kindly provided by J. Schlessinger). Starved or EGF-stimulated F19-91 cells were lysed in RIPA buffer and mixed with the corresponding bacterial MBP fusion protein by gentle inversion for 3 h at 4°C. Complexes were recovered by centrifugation, washed four times and analysed by immunoblotting with anti-P-Tyr or anti-EGF receptor antibodies.

Whether these proteins precipitated because of their association with the Vav SH2 domain or to the activated EGF receptor remains to be determined.

Activation of the T-cell receptor (TCR) also results in the induction of signal transduction pathways that involve tyrosine phosphorylation⁵. We therefore investigated whether the tyrosine residues on endogenous p95^{vav} in T cells become phosphorylated during T-cell activation by treating CEM cells with monoclonal antibodies against components of the T-cell receptor (anti-CD3 antibodies) and the auxiliary CD4 antigen. No significant phosphorylation of p95^{vav} tyrosine residues was observed either in unstimulated cells or in cells incubated with anti-CD3 or crosslinked anti-CD4 monoclonal antibodies (Fig. 4a). But when CEM cells were activated with crosslinked anti-CD3 $\times$ CD4 antibodies, tyrosine phosphorylation in p95^{vav} was induced (Fig. 4a). Results were similar from two other human T-cell lines, HBP-ALL and Jurkat, although Jurkat cells had significant levels of tyrosine-phosphorylated p95^{vav} even before stimulation (data not shown).

To define the timing of p95^{vav} tyrosine phosphorylation through the signalling process mediated by the TCR/CD4 antigen complex, we stimulated CEM cells with crosslinked anti-CD3 $\times$ CD4 monoclonal antibodies for different times. As shown in Fig. 4b, tyrosine phosphorylation of p95^{vav} molecules could be readily detected within 30 seconds of TCR/CD4 activation, reaching a maximum after 1 min and decreasing thereafter.

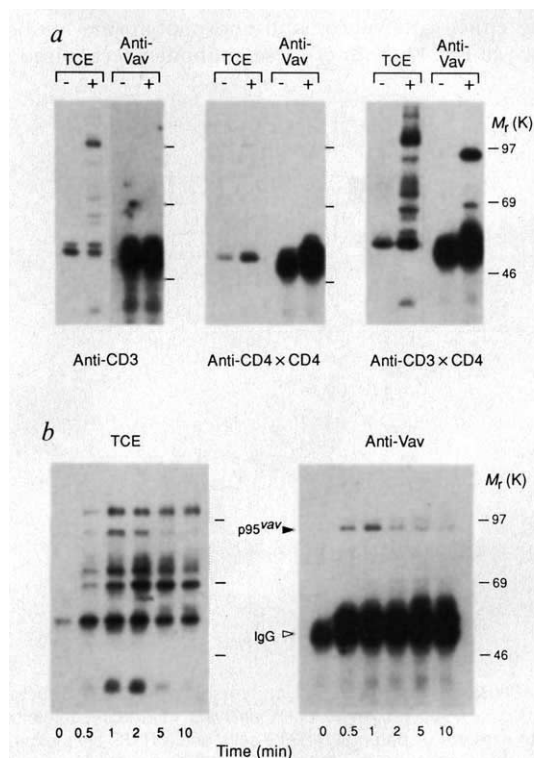


FIG. 4 *a*, Anti-P-Tyr immunoblot analysis of total cellular extracts (TCE) and anti-Vav p95^{vav} immunoprecipitates obtained from CEM cells stimulated by anti-CD3, crosslinked anti-CD4 and crosslinked anti-CD3 $\times$ CD4 monoclonal antibodies. *b*, Anti-P-Tyr western blot of total cellular extracts and anti-Vav p95^{vav} immunoprecipitates obtained from CEM cells stimulated by crosslinked anti-CD3 $\times$ CD4 monoclonal antibodies for the indicated times.

METHODS. Human CEM cells (ATCC TIB 195) were maintained in RPMI 1640 medium containing 10% fetal calf serum. Cells were collected by centrifugation, resuspended at 2×10^7 cells per ml in prewarmed RPMI medium and stimulated at 37°C for 1 min (*a*) or for the indicated times (*b*) with the corresponding anti-CD3, crosslinked anti-CD4 or crosslinked anti-CD3 $\times$ CD4 monoclonal antibodies²¹. Samples corresponding to total cellular extracts were derived from 5×10^4 cells. Immunoprecipitates were obtained from *a*, 5×10^7 cells and *b*, 2×10^7 cells.

Comparison of the kinetics of p95^{vav} tyrosine phosphorylation with those of other tyrosine-phosphorylated molecules indicates that p95^{vav} is one of the earliest proteins to be phosphorylated in response to the activation of the TCR complex in CEM cells (Fig. 4b).

Our results and those reported in the accompanying manuscript⁶ indicate that p95^{vav} is a downstream component of the signalling process initiated by activation of the TCR. These phosphorylation events are likely to be mediated by tyrosine protein kinases of the *src* gene family. Crosslinking of CD4 or CD8 cell-surface molecules results in the activation of p56^{lck} (refs 7, 8). The same kinase becomes activated in interleukin-2(IL-2)-stimulated T cells^{9,10} and seems to be physically associated with the IL-2 receptor⁹. Moreover, p59^{lyn} interacts with the TCR complex¹¹. Finally, T cells express additional tyrosine kinases such as p62^{c-yes} (ref. 7). It will be important to determine whether p95^{vav} is phosphorylated by any of these enzymes or is a substrate for an as-yet-unidentified tyrosine protein kinase.

The identification of p95^{vav} as a substrate for tyrosine protein kinases may lead to the identification of the cascade of events that translates tyrosine phosphorylation into nuclear events. Unlike other tyrosine kinase substrates implicated in signal transduction, p95^{vav} contains no structural motifs known to be associated with enzymatic activity. In this regard, p95^{vav} resembles the product of another proto-oncogene, *c-crk* (ref. 12), and it contains motifs so far unknown in other tyrosine protein kinase substrates. These include a helix-loop-helix (HLH)-like domain followed by an imperfect leucine zipper, an acidic region, two putative nuclear-localization signals and a zinc-finger-like structure homologous to the phorbol ester-binding domains of protein kinase C (refs 2, 3). Similar cysteine-rich motifs have been identified in diacylglycerol kinase, c-Raf and n-chimerin¹³⁻¹⁵. Interestingly, the HLH-like domain of p95^{vav} is also related to those of Myc proteins (42% homology, with 30% identity) and to that of Max (38% homology, with 20% identity)¹⁶⁻¹⁸. It is possible that p95^{vav} can interact with either Myc or Myc-related proteins containing HLH/leucine-zipper motifs. The HLH-like motif must regulate p95^{vav} activity as deletion of these sequences results in its oncogenic activation¹⁻³. Therefore the HLH/leucine zipper domain of p95^{vav} may mediate its interaction with downstream signal transduction elements, some of which might be implicated in transcriptional events.

Tyrosine phosphorylation seems to be a widely used signal transduction mechanism in cells of most if not all haematopoietic lineages⁷, so the possible participation of p95^{vav} in the tyrosine phosphorylation cascade may not be restricted to T cells. Indeed, p95^{vav} is phosphorylated at tyrosine residues upon activation of the IgE receptor in the rat basophilic leukaemia cell line RBL-2H3 (ref. 6). Similar results have been obtained in the mast cell-like RBL-2H3 and PT18 cell lines and in B lymphocytes (unpublished results). These observations indicate that the *vav* gene may be important in haematopoietic signal transduction. □

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Tyrosine phosphorylation of *vav* proto-oncogene product containing SH2 domain and transcription factor motifs

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ACTIVATION of receptor-linked and cytoplasmic protein tyrosine kinases is crucial in the control of normal and abnormal cell growth and differentiation^{1,2}. Some substrates of protein tyrosine kinases such as phospholipase C γ and *ras* GTPase-activating protein (GAP) contain sequences homologous to the *src* protein domains SH2 and SH3 (refs 3-9). The proto-oncogene *vav* is expressed in haematopoietic cells and its product Vav contains sequence motifs commonly found in transcription factors, such as helix-loop-helix, leucine-zipper and zinc-finger motifs and nuclear localization signals¹⁰⁻¹², as well as a single SH2 and two SH3 domains. Here we show that stimulation of T-cell antigen receptor on normal human peripheral blood lymphocytes or on human leukaemic T cells, and the crosslinking of IgE receptors on rat basophilic leukaemia cells, both promote the phosphorylation of tyrosine residues in Vav. Moreover, activation of the receptor for epidermal growth factor leads to marked tyrosine phosphorylation of Vav in cells transiently expressing *vav*, and Vav associates with the receptor through its SH2 domain. We propose that *vav* encodes a new class of substrates whose tyrosine phosphorylation may provide a mechanism for direct signal transduction linking receptors at the cell surface to transcriptional control.

A schematic representation of the primary structure of Vav illustrating the relative position of the putative structural motifs and *src* homologous domains SH2 and SH3 is illustrated in Fig. 1a. As many proteins that contain both SH2 and SH3 domains are substrates of tyrosine kinases, we investigated the phosphorylation state of Vav in two cell lines: Bac1.2F5 is an SV40-immortalized murine macrophage cell line¹³ which expresses endogenous proto-*vav*, and K62 is an NIH3T3 cell line transfected with the full-length human *vav* proto-oncogene. Vav was found to be constitutively phosphorylated on tyrosine and serine residues in both cell lines, whereas phosphothreonine was detected in only trace amounts (Fig. 1b, c, d). As *vav* seems to be normally expressed in cells of all haematopoietic lineages¹⁰, we have examined the phosphorylation of Vav in lymphocytes after activation of the T-cell antigen receptor (TCR) with monoclonal antibodies. As with stimulation by antigen, these antibodies activate the TCR to induce tyrosine kinase activity¹⁴ and the activation and tyrosine phosphorylation of phospholipase C γ

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in intact cells^{15,16}. Stimulation of Jurkat cells or normal T cells with anti-TCR monoclonal antibodies led to the appearance of several tyrosine phosphoproteins in lysates from these cells (Fig. 2a and b, lanes 1 and 2). Among the tyrosine phosphoproteins induced in Jurkat (Fig. 2a, lanes 7 and 8) and peripheral lymphocytes is phospholipase C (PLC)- γ 1. Basal tyrosine phosphorylation of Vav was detected in unstimulated Jurkat cells and in the peripheral blood lymphocytes, but was greatly increased by TCR stimulation (Fig. 2a and b, lanes 5 and 6).

We also looked at tyrosine phosphorylation of Vav in another haematopoietic lineage using the rat basophilic leukaemia cell line, RBL-2H3. In this mast cell model system, the abundant Fc ϵ R1 is primed by IgE binding and activated by crosslinking the Fc ϵ R1-IgE complexes with multimeric antigen^{17,18}. Stimulation of RBL-2H3 cells leads to tyrosine phosphorylation of a variety of cellular proteins^{19,20} (W.L., B.M., J.S. and J.M.O., manuscript submitted). When these cells were stimulated with antigen, the phosphorylation of Vav tyrosines was dramatic (Fig. 2c), demonstrating not only that phosphorylation can occur under physiological conditions but also that phosphorylation of Vav may be important in mast cell signal transduction.

It has been proposed that tyrosine phosphorylation resulting from stimulation by IgE and TCR is mediated by cytoplasmic tyrosine kinases of the *src* family^{14,21}, but exactly which tyrosine kinase(s) is responsible for Vav phosphorylation in mast cells and T cells is not known. In contrast, the interaction between

growth-factor receptors having tyrosine kinase activity, such as epidermal growth factor receptor (EGFR) and platelet-derived-growth-factor receptor, and SH2/SH3-containing proteins is well established^{9,22-24}. We therefore studied the tyrosine phosphorylation of Vav by EGFR.

We used a transient expression system in 293 cells to examine the interaction of Vav with the EGFR. We found that in this system *vav* expression was less than in the stably transfected NIH3T3 cells and constitutive tyrosine phosphorylation was lower. When these cells were stimulated by epidermal growth factor (EGF), tyrosine phosphorylation of Vav was observed owing to the activation of endogenous EGFR in the 293 cells (Fig. 3a, c). As shown in Fig. 3a, tyrosine phosphorylation of Vav was detected in lysates directly immunoblotted with anti-phosphotyrosine antibodies, indicating that Vav can act as an EGFR substrate. Cotransfection of EGFR complementary DNA with *vav* led to EGFR overexpression and phosphorylation of Vav was increased (Fig. 3c). Antibodies against Vav coimmunoprecipitated a tyrosine-phosphorylated protein (migrating at a position corresponding to a relative molecular mass (M_r) of 170,000) from cells cotransfected with *vav* and EGFR, but not from cells transfected with either on its own (Fig. 3c). The band of M_r 170,000 (170K) comigrates with the EGFR and corresponds to associated Vav and EGFR, as verified by immunoblotting with anti-EGFR antibodies.

We confirmed this association of Vav with EGFR *in vitro* by immunoprecipitating Vav from *vav*-transfected NIH3T3 cells,

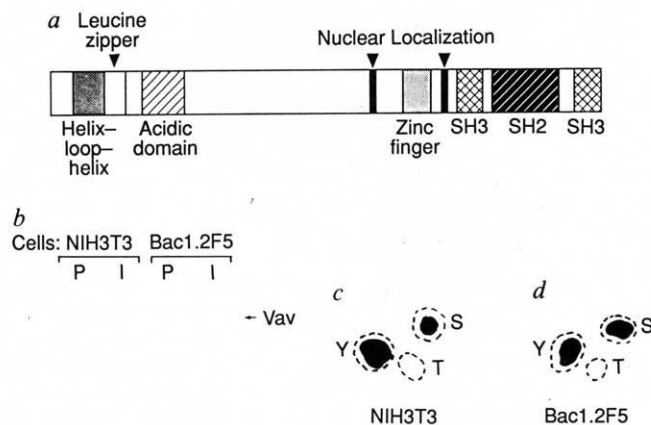


FIG. 1 The product of the proto-oncogene *vav* is constitutively tyrosine-phosphorylated in Bac1.2F5 cells and in *vav*-transfected NIH3T3 cells. a, Schematic representation of Vav indicating the relative size and position of the different structural motifs. b, After labelling with 1 mCi ml⁻¹ of [³²P]orthophosphate for 2 h, Vav was immunoprecipitated with preimmune (P) or immune (I) sera from quiescent NIH3T3 cells which overexpress *vav*¹¹ or from the SV40-immortalized macrophage cell line Bac1.2F5 (ref. 13). c and d, Phosphorylated Vav bands were cut from the gel slice, eluted and analysed for phosphoaminoacids (Y, Tyr; T, Thr; S, Ser).

METHODS. NIH3T3 (ref. 29) and Bac1.2F5 (ref. 30) were grown as before. Quiescent NIH3T3 cells were preincubated in phosphate-free medium containing 1% dialysed fetal calf serum (Gibco/BRL) for 2 h before labelling with 1 mCi ml⁻¹ of [³²P]orthophosphate (NEN/Dupont). Bac1.2F5 cells were deprived of CSF-1 for 24 h and then treated like NIH3T3 cells, except that 5% and 2.5% dialysed serum were used for preincubation and labelling, respectively. Cell lysates were immunoprecipitated overnight at 4 °C with anti-Vav antibodies, washed extensively with ice-cold RIPA buffer, and separated by 8% SDS-PAGE. Bands corresponding to Vav were excised from gels and analysed for phosphoaminoacids as described³¹ but with minor modifications. Briefly, excised bands were rehydrated in 50 mM NH₄HCO₃ and homogenized, and the proteins extracted overnight at 37 °C with 5% β -mercaptoethanol, 0.1% SDS then precipitated in 20% trichloroacetic acid, washed once with ice-cold acetone and 3 times with water, and hydrolysed in 100 μ l 5.7 N HCl for 1 h at 110 °C. The hydrolysate was washed several times with water, dried, and resuspended in 5 μ l running buffer, pH 1.9, containing 1 mg ml⁻¹ phosphoaminoacid standards. Samples were spotted on cellulose TLC plates and subjected to two-dimensional electrophoresis. Plates were dried, sprayed with 0.5% ninhydrin in acetone, incubated at 56 °C for 10 min, and exposed to film.

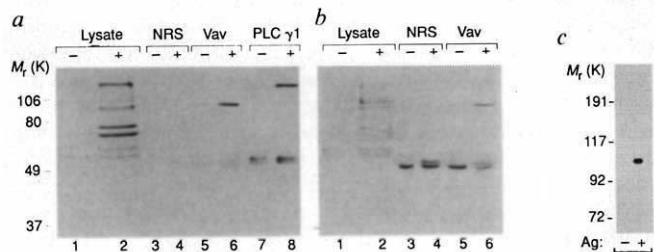


FIG. 2 Stimulation of the TCR and Fc ϵ R1 induces an increase in the tyrosine phosphorylation of Vav in T cells and RBL-2H3 cells, respectively. Jurkat cells (a) or nonadherent peripheral blood lymphocytes (b) were stimulated with anti-TCR V β or anti-CD3 monoclonal antibodies, respectively, for 2 min; lysates were immunoprecipitated with normal rabbit serum (NRS; lanes 3, 4); anti-Vav serum (lanes 5, 6); or anti-PLC γ 1 monoclonal antibodies (lanes 7, 8). Immunoblots of lysates (lanes 1, 2) or immunoprecipitates from cells stimulated with anti-TCR or medium (+/-) were probed with an anti-phosphotyrosine antibody. c, IgE-primed RBL-2H3 cells ($\sim 1 \times 10^7$) were incubated for 2 min with (+) and without (-) the antigen (Ag), Dinitrophenylated bovine serum albumin (DNP-BSA), immunoprecipitated with anti-Vav antibodies, separated on a 7% SDS-polyacrylamide gel and immunoblotted with anti-P-Tyr antibodies. Basal tyrosine phosphorylation of Vav was detected in the RBL-2H3 cells after longer exposure of this and other autoradiograms.

METHODS. a and b, Jurkat human leukaemic T cells (2×10^7) or nonadherent normal peripheral blood lymphocytes resuspended (3.3×10^7) in 100 μ l medium were stimulated at 37 °C for 2 min with saturating quantities of anti-V β monoclonal antibody (C305; ref. 16) or anti-CD3 (A32.1; ref. 32), plus rabbit anti-mouse IgG, respectively. Cells were washed in ice-cold medium and lysed in 200 μ l Tris-buffered saline containing 1% Nonidet P-40 and phosphate inhibitors¹⁶. Immunoprecipitates were isolated using protein A-coupled Sepharose beads precubated with normal rabbit serum, rabbit anti-Vav serum or a pool of anti-PLC γ 1 monoclonal antibodies. Immunoblots were prepared and analysed with the 4G10 anti-phosphotyrosine antibody³⁴ as described¹⁶. c, RBL-2H3 cells were grown in minimum essential medium (MEM) with 15% fetal calf serum and their Fc ϵ R1 primed with 1 μ g ml⁻¹ anti-DNP IgE (ref. 35). For stimulation, cells were scraped off, washed to remove excess IgE, and resuspended in MEM with 15% fetal calf serum to $\sim 2 \times 10^7$ cells ml⁻¹. Aliquots (2 ml) were then incubated with antigen, 0.1 μ g ml⁻¹ DNP-BSA, for 2 min at 37 °C. The reaction was stopped by the addition of 7.5 ml ice-cold medium, the cells pelleted at 4 °C and lysed in a 1% Triton X-100 buffer, immunoprecipitated with anti-Vav and immunoblotted with anti-phosphotyrosine antibodies⁵.

incubating it with lysates from NIH3T3 cells that overexpressed EGFR and immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 4a, anti-Vav immunoprecipitates associate with a 170K tyrosine-phosphorylated protein, the autophosphorylated EGFR. As found for other signalling molecules, the SH2 domain of Vav is sufficient to mediate binding to the tyrosine-phosphorylated EGFR (Fig. 4b). We also found that the soluble cytoplasmic kinase domain of the EGFR²⁴ can directly phosphorylate tyrosine on immunoprecipitated Vav *in vitro* (results not shown). These results indicate that, like other proteins containing the SH2 domain, Vav binds to activated EGFR and can be tyrosine-phosphorylated by the receptor both *in vitro* and in living cells.

This work and an accompanying report²⁵ show that Vav is tyrosine-phosphorylated in response to growth factors, IgE-receptor crosslinking, and TCR activation and it links SH2 domains with tyrosine phosphorylation as has been shown for other substrates of tyrosine kinases³⁻⁹. It may be that the high-affinity binding of SH2 domains to autophosphorylated tyrosine kinases facilitates tyrosine phosphorylation of these bound proteins²²⁻²⁴. Indeed, we have demonstrated EGF-induced tyrosine phosphorylation of Vav and association of Vav with the activated EGFR through its SH2 domain. EGFR is not considered to be a haematopoietic receptor as such, though it is expressed in avian erythrocyte precursors²⁶. However, we could not detect tyrosine phosphorylation of Vav induced by colony-stimulating factor-1 in murine macrophage cells expressing receptors for this factor (S.K. *et al.*, unpublished results).

Because it contains a helix-loop-helix-like motif, a zinc-finger and an incomplete leucine-zipper motif, Vav may be involved in the regulation of transcriptional activation. There is no definitive evidence, however, that Vav is a transcriptional

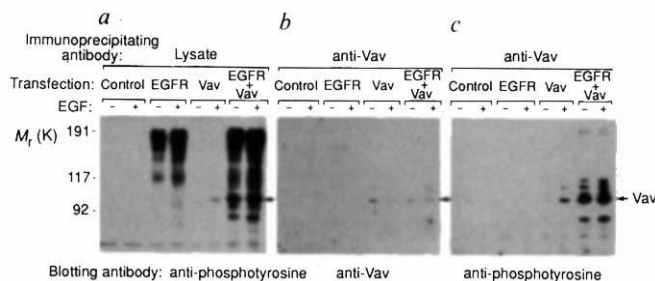


FIG. 3 Vav is tyrosine-phosphorylated after stimulation of intact cells by EGF. Embryonic kidney cells (293) were transiently transfected with human EGFR complementary DNA or *vav* proto-oncogene, or both together, or with control vector. Cells were then stimulated with EGF (40 nM) for 2 min at 37 °C and lysed in 1% Triton X-100 buffer. **a**, Sample buffer was added directly to lysates (250 µg protein) and immunoblotted with anti-phosphotyrosine antibodies. **b**, Lysates (500 µg) were immunoprecipitated with anti-Vav and immunoblotted with anti-Vav. **c**, Lysates (2 mg) were immunoprecipitated with anti-Vav serum and immunoblotted with anti-phosphotyrosine. Tyrosine phosphorylation of the transfected EGFR occurs both in the presence and absence of EGF. This is probably due to the high level of expression of EGFR which leads to ligand-independent stimulation of the tyrosine kinase activity. In the transfected 293 cells, *vav* was expressed mainly as a protein of M_r 95K, although in some cell systems other forms of Vav have been found¹². These forms are recognized by anti-Vav antibodies and are also tyrosine-phosphorylated. The modification(s) that give rise to the higher M_r forms of Vav in these cells are not known, but their abundance is not affected by tyrosine phosphorylation.

METHODS. Embryonic human kidney 293 cells were transfected using precipitation with calcium phosphate³⁶ with a eukaryotic expression vector based on a cytomegalovirus promoter and containing human *proto-vav*, or human EGFR. After transfection for 24 h, cells were washed and maintained in 1% fetal calf serum overnight, then stimulated with 40 nM EGF for 2 min at 37 °C. Lysates were normalized for protein content, immunoprecipitated with anti-Vav antibody and immunoblotted with antiphosphotyrosine or anti-Vav antibodies as described for Fig. 2.

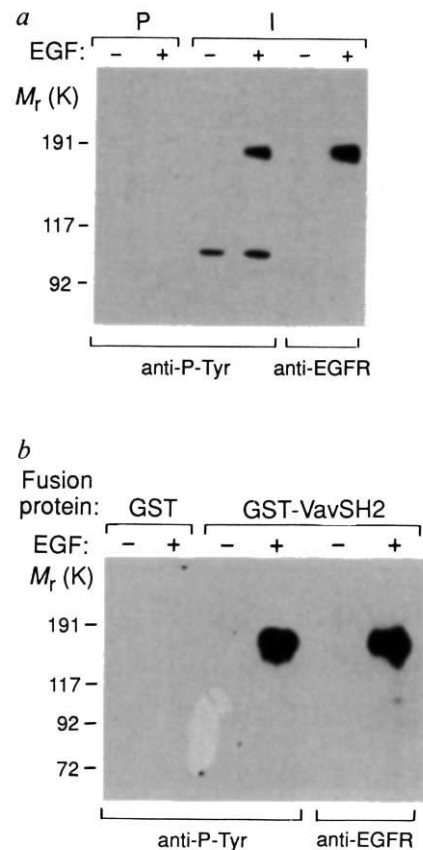


FIG. 4 Binding of Vav and the Vav SH2 domain to tyrosine-phosphorylated EGFR. **a**, Vav was immunoprecipitated from NIH3T3 cells overexpressing *vav* with immune (I) or preimmune (P) serum and mixed with lysates from EGF-stimulated or unstimulated NIH3T3 cells overexpressing EGFR (HER14 cells²⁹). Proteins were then separated by SDS-PAGE and immunoblotted with either antiphosphotyrosine (anti-P-Tyr) or anti-EGFR antibodies. **b**, Glutathione S-transferase (GST) fused to Vav SH2 domain or GST alone were immobilized on glutathione-agarose and EGFR-containing lysates were bound and immunoblotted as in **a**.

METHODS. **a**, Lysates from NIH3T3 cells overexpressing *vav* were immunoprecipitated with anti-Vav antibodies, washed three times with HNTG (20 mM HEPES, pH7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) and incubated with lysates from 1×10^7 NIH3T3 cells overexpressing EGFR for 90 min at 4 °C. Bound proteins were washed three times with HNTG, separated by electrophoresis on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted. **b**, Oligonucleotide primers flanking the *vav* SH2 domain and containing appropriate restriction sites were synthesized and used to amplify a DNA fragment from a human *vav* cDNA template. The fragment was cleaved with *Bam*HI and *Eco*RI and subcloned into *Bam*HI/*Eco*RI-cleaved pGEX3X (Pharmacia). Fusion proteins were purified on a large scale by affinity chromatography on glutathione-agarose beads³⁷. The GST-VavSH2 fusion protein contains residues 663-776 of human Vav. About 5 µg GST or GST-VavSH2 fusion protein was bound to EGFR-containing lysates as in **a**.

activator or that it binds DNA. But it is possible that Vav regulates the action of transcription factors by formation of heterodimers through its helix-loop-helix and leucine-zipper domains in much the same way as other proteins with these motifs, for example the *myc* and *max* proteins^{27,28}. Immunofluorescence microscopy using anti-Vav antibodies indicates that after stimulation of mast cells, Vav is localized both in the cytoplasm and in the nucleus at 2, 20 or 120 min after activation of the IgE receptor (data not shown). It will be interesting to look for similar proteins containing typical nuclear motifs and SH2 domains that are tyrosine-phosphorylated in response to stimulation by growth factor in haematopoietic cells and other lineages. Vav may be a prototype for a new class of proteins

that can relay signals directly from the plasma membrane to modulate transcriptional events. □

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Kinesin-related cut7 protein associates with mitotic and meiotic spindles in fission yeast

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SEVERAL mitotic and meiotic gene products are related to the microtubule motor kinesin, providing insight into the molecular basis of the complex motile events responsible for spindle formation and function¹. Of these genes, three have been shown to affect spindle structure when mutated^{2–6}. The most severe phenotype is seen in *Aspergillus nidulans bimC* and *Schizosaccharomyces pombe cut7* mutants. In both fungi the intranuclear spindle is bipolar, with microtubules that emanate from spindle pole bodies at either pole, interdigitating in a central overlap zone. In *bimC* and *cut7* mutants, microtubule interdigitation does not appear to take place, instead two unconnected half spindles form and chromosome separation fails^{2,6,7}. Here we report that *cut7* protein concentrates on

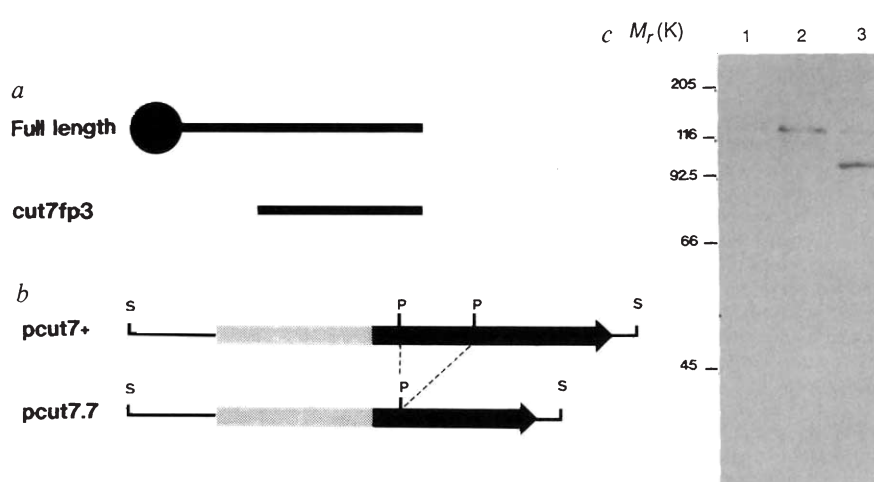
or near the spindle pole bodies throughout mitotic and meiotic nuclear division and associates with mitotic spindle microtubules in a stage-specific manner, associating with the mid-anaphase B midzone. In *cut7^{ts}* mutants, spindle pole bodies stain but mitotic microtubules do not.

The *cut7* polypeptide contains an N-terminal, kinesin heavy-chain gene motor domain homologous region and a C-terminal 'tail' that lacks significant homology to previously identified proteins (Fig. 1a)^{1,6}. Two polyclonal antisera were raised against, and affinity-purified with, a fusion protein (*cut7fp3*) containing the 473 C-terminal residues (Fig. 1a). Both recognize a band of relative molecular mass 132,000 (*M_r* 132 K) on blots whose intensity is enhanced in strains harbouring the *cut7⁺* gene multicopy plasmid *pcut7⁺* (formerly pSKa78 (ref. 6)) (Fig. 1b, c; lane 2) and an additional 108K band in strains harbouring a plasmid bearing a truncated *cut7* gene (*pcut7-7*, Fig. 1b, c; lane 3).

Both antibodies give similar immunofluorescence patterns, recognizing a single structure, identified by comparison with wild-type microtubule distribution⁸ as the mitotic spindle. Spindle poles stain strongly throughout mitosis, whereas the central spindle stains with stage-dependent variation in signal intensity and distribution. Central staining is comparatively

FIG. 1 Antibody creation and characterization. a, Diagram depicting *cut7* and the *cut7fp3* fusion protein. The 400-amino-acid kinesin heavy-chain head domain homologous region is represented by a circle, the remainder of the molecule by a thick black line. b, The inserts of the plasmids: *pcut7⁺* (formerly pSKa78; ref. 6) and *pcut7-7*. *pcut7-7* is derived from *pcut7⁺* by deleting an inframe 597-base-pair (bp) internal *Pst*I fragment. The predicted *M_r* of *pcut7-7* gene product is 100K. c, A protein blot using affinity-purified antisera AP5LB. Each lane contains yeast extracts made from 10⁷ *leu1.32 h⁻* cells carrying a 2-μm-based LEU2-carrying plasmid (this gene complements the *leu1.32* mutation in the host strain and ensures plasmid maintenance when strains are grown without leucine). Strains containing the following plasmids are shown. Lane 1, pSK248, the vector used to construct plasmids *pcut7⁺* and *pcut7-7*; lane 2, *pcut7⁺*; lane 3, *pcut7-7*. The intensity of the 132K band in lane 1 is increased when the *cut7⁺* gene is present on a multicopy number plasmid. An additional 108K strong band is seen when cells carry a smaller *cut7-7* gene on a multicopy plasmid.

METHODS. The *pcut7⁺* 2.3-kilobase (kb) *Bgl*II–*Bam*H1 fragment was ligated



in-frame to the T7 gene 10 gene²¹ to produce plasmid *pcut7fp3*. Fusion protein production²¹, antisera generation²², immunoblotting²² and affinity purification²³ were done as described previously. Yeast were cultivated in appropriately supplemented EMM2 (ref. 24) at 33 °C.

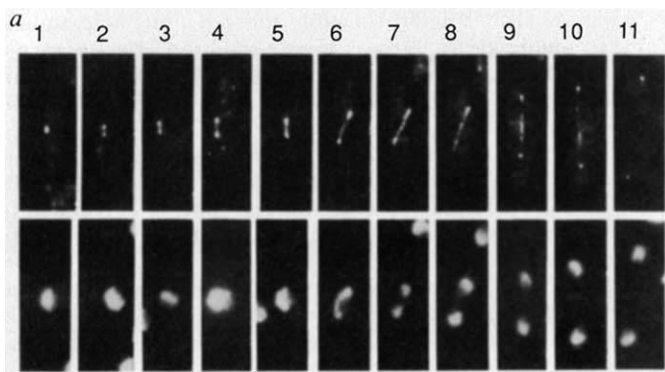
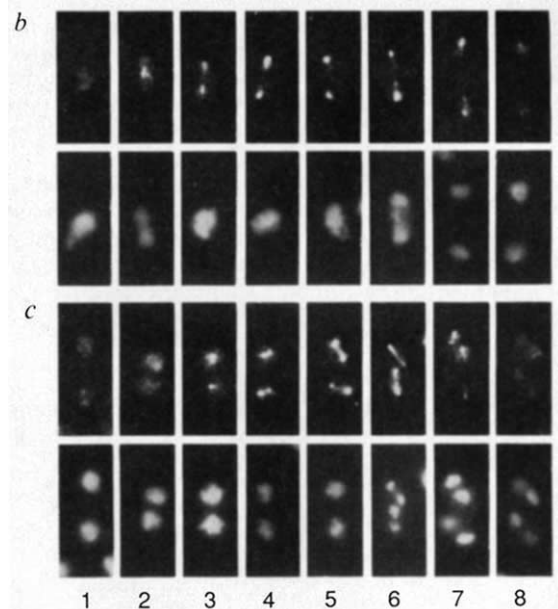


FIG. 2 Cut7 staining using antibody preparation cAP5LB in mitotic (a) and meiotic nuclear divisions (b, c). For each stage of division the upper panel shows typical cAP5LB staining, the lower the corresponding DAPI staining of chromatin of the same cell²⁵. Images of individual cells from an asynchronous culture are arranged according to their relative progression through nuclear division. a, The mitotic spindle stained as described in the text. Alternative fixation with -20°C methanol and use of different anti-cut7 antibodies gave similar results (data not shown). b, As cells enter meiosis, chromatin undergoes a series of characteristic changes²⁶. Cut7 staining initially appears as a diffuse chromatin-associated stain as cells form the 'horse tail' structure²⁶ in early meiosis. Subsequently a bright dot of cut7 staining appears on spindle formation (panel 2) before the establishment of a metaphase meiosis I spindle with a chromosomal plate and strong polar cut7 staining (panels 3–5). The chromosomes move to the poles (panel 6) before anaphase B (panel 7 and 8). As the nuclear envelope remains intact throughout nuclear division, diffuse nuclear staining is seen as the cut7 protein diffuses away after depolymerization of the first meiotic spindle, before the formation of the second meiotic spindle (c, panel 1). Cut7 antibodies also stain the meiosis II spindle (c). Central spindle staining is stronger in meiosis II (c) than meiosis I, but neither show the midzone



staining typical of mitosis (a) possibly underlining functional differences between meiotic and mitotic spindles. Scale bar, $5\mu\text{m}$.

METHODS. a, Haploid *leu1.32* h^{-} cells were grown in rich medium at 33°C (ref. 24). b, c, An *ade6.M210/ade6.M216 ura4.d18/ura4.d18 leu1.32/leu1.32 his2.245/+* diploid was grown in rich medium, washed in SPL (ref. 27), resuspended in SPL at 33°C for 10 h before immunofluorescence. Immunofluorescence microscopy used formaldehyde and glutaraldehyde fixation⁸. After affinity purification²³, antibodies were concentrated (cAP5LB) using a Sartorius centriscat I tube (cut-off 20,000) and diluted to 10% in PEMBAL 100 mM PIPES, 1 mM EGTA, 1 mM MgSO_4 , 1% BSA, 0.1% NaN_3 , 100 mM lysine-HCl, pH 6.9.

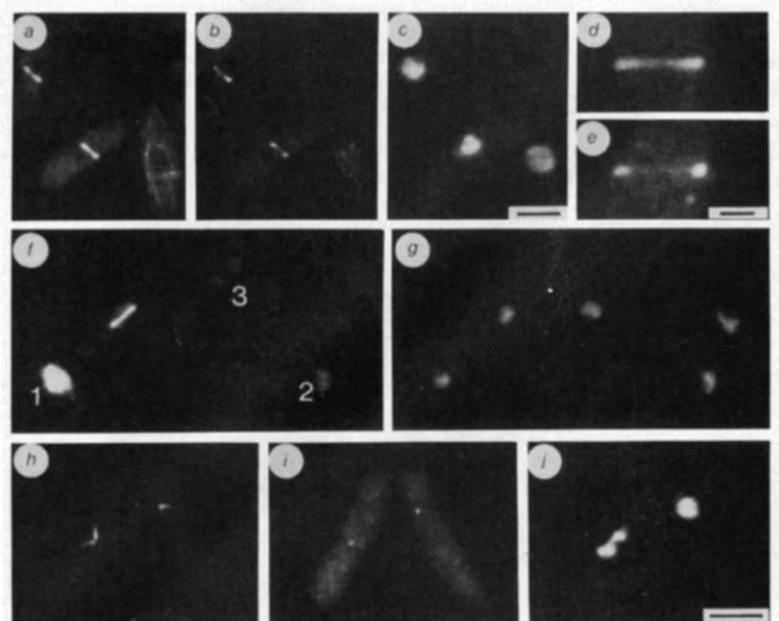
weak until spindle elongation (Fig. 2b, c, panels 2–6) whereupon its intensity transiently rivals polar staining (Fig. 2b, c, panel 8) when it is punctate, possibly reflecting interaction with some nodular⁹ or regular spindle structure. On further elongation, antibodies stain the mid-zone (Fig. 2a, panel 9) before spindle staining disappearance at or before maximal elongation (Fig.

2a, panel 11). No signal is seen in wild-type interphase cells. Cut7 also associates with the functionally distinct meiosis I, reductive, and meiosis II, equational, spindles (Fig. 2b, c).

The *nuc2.633* mutant cells arrest cell-cycle progression with a metaphase spindle owing to a temperature-sensitive (ts) lesion in the *nuc2* protein¹⁰. The cut7 staining in arrested *nuc2* cells

FIG. 3 Cut7 localization in different genetic backgrounds. Arrest of *nuc2.633* mutant cells¹⁰ was at 36°C for 3 h, and stained by immunofluorescence with a, YOL1/34, anti-tubulin²⁸; b, cAP5LB, anti-cut7 antibodies; and c, DAPI²⁵. Polar cut7 staining is stronger than in the central spindle. Comparison of enlarged anti-tubulin (d) and cAP5LB (e) images of the same cell shows that cut7 staining is coincidental with, or distal to, the end of the anti-tubulin staining. On the introduction of the *cut7+* multicopy plasmid, *pcut7+* into wild-type cells, stronger mitotic spindle and interphase nuclear staining was observed (f, cAP5LB; g, DAPI). The level of cut7 staining varied, consistent with the variable copy number of 2- μm -based vectors in *S. pombe*: cell 1, presumably having the highest copy number, stains strongly; 2, lower copy number; and 3, no detectable difference from wild type. In cell 2, cut7 staining is predominantly in the chromosomal domain. In addition chromosome disjunction and spindle defects could be observed in the most brightly staining mitotic cells (data not shown). h–j, *ts- cut7.446* cells stained with h, anti-tubulin TAT1²⁹; i, cAP5LB; j, DAPI. Although spindle-pole-body associated staining is observed, microtubule association is absent in *cut7.446* and *cut7.24* mutants and is greatly reduced in a third allele (*cut7.23*) (data not shown). Scale bars, a–c, f–j, $5\mu\text{m}$; e, $1\mu\text{m}$.

METHODS. *pcut7+* bearing cells were grown to mid-log phase in EMM2²⁴. Mutant cells were grown to early log phase in rich medium at 25°C , shifted to 36°C for 3 h and fixed for immunofluorescence⁸. For cut7/tubulin double immunofluorescence, cells were incubated with 10% cAP5LB antibodies followed by 0.5% sheep anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Cappel) before incubation in 1% YOL 1/34 (ref. 28) (a and d).



or 10% TAT1 culture supernatant²⁹ (h) and subsequent incubation in the relevant tetramethyl-rhodamine-isothiocyanate-conjugated secondary antisera.

is indistinguishable from wild-type metaphase staining (Fig. 3b, e), confirming that the anaphase increase in central spindle association is not a function of the time since mitotic initiation, but requires cell-cycle transition. Comparing *nuc2^{ts}* tubulin and *cut7* images (Fig. 3d, e) suggests that *cut7* is adjacent to, or on, the spindle pole bodies.

Although wild-type interphase cells do not show *cut7* staining, those carrying *pcut7⁺* can show strong nuclear fluorescence (Fig. 3f, g, cell 1). Along with the mutant phenotype⁶ and previous ultrastructural analysis^{9,11}, the lack of interphase cytoplasmic and mitotic astral microtubule staining, even after such over-expression, suggests that, unlike kinesin¹²⁻¹⁵, *cut7* participates

in intranuclear spindle function and not other microtubule-based motile events such as vesicle transport. The absence of equatorial microtubule organizing centre (MTOC)^{8,16} staining indicates that *cut7*, unlike γ -tubulin, is not a general MTOC component¹⁶.

In arrested *cut7^{ts}* mutant cells^{6,7}, spindle pole body-associated staining is preserved but microtubule staining is absent (Fig. 3h-j), indicating that although full *cut7* function is required for microtubule association, it is not required for localization of spindle pole bodies.

What is the role of *cut7*? Half-spindle interdigitation seems to be defective in *cut7^{ts}* mutants⁶ (Fig. 3h), indicating *cut7* is essential for, at least, this process. It is not clear whether it is also essential for sliding apart half spindles. This may be driven by *cut7* alone, additional mitotic spindle motors, forces created by extranuclear microtubules pulling the nuclei apart¹⁷, or any combination of these factors, some of which may be redundant.

If there prove to be other fission yeast spindle motors, will they be essential for spindle formation? If not, why is *cut7* required for spindle formation? The answer may lie in the difference between realigning parallel microtubule arrays into an antiparallel lattice (Fig. 4a, steps I, II) and sliding apart such a lattice (Fig. 4a, steps III, IV). Microtubules have an intrinsic polarity with the 'plus end' generally distal to the MTOC. Motors generally direct microtubule movement to one end. Therefore, forces optimal for sliding apart antiparallel arrays would be ineffective in realigning parallel ones and could even end up 'zipping' them together. Thus realignment may require a specialized motor activity, achieved by specific motor localization, biochemical properties, oligomerization or tail structure. In this context, polar *cut7* may be important for spindle formation and functionally distinct from central spindle *cut7*.

Understanding *cut7* function requires determination of its assumed motile properties, as different kinesin-related molecules direct microtubule movement in opposite directions^{1,18,19}. But *cut7* localization to the midzone, where plus-end directing motors have been proposed to reside²⁰, suggests it may possess such activity. We propose two models in which a spindle-pole-body-associated, plus-end-directing *cut7* reorients the microtubule arrays during spindle formation (Fig. 4b, c). □

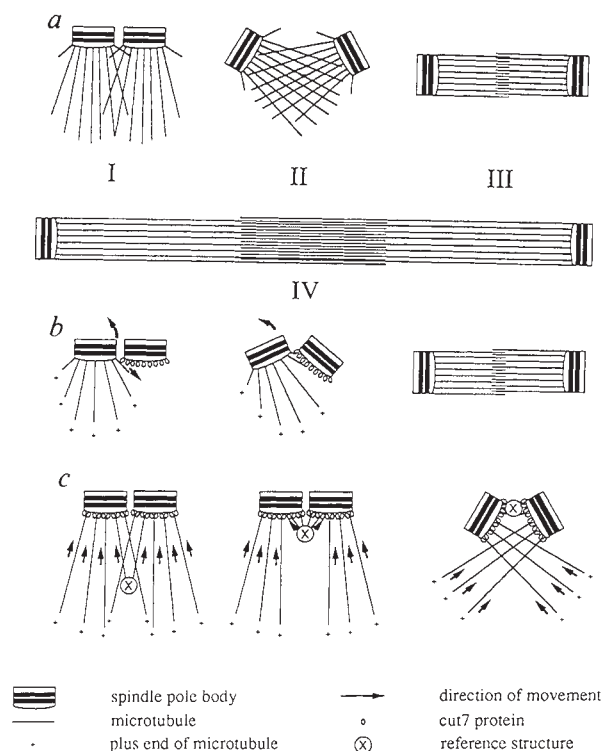


FIG. 4 Models. *a*, The basis of spindle formation. Spindle formation in *S. pombe* requires interdigitation of two initially predominantly parallel microtubule arrays. The two half spindles undergo coincidental separation and reorientation¹¹. *b*, *c*, Order of events is from left to right. *b*, Spindle pole body (SPB) reorientation results from SPB-associated *cut7*-based expulsion of the opposite pole. Polar *cut7* (ovals) directs movement towards the free plus end (SPB distal) of any microtubules from the sister SPB²⁰. Lateral association results in two forces, one causing that pole body to move down the microtubule and one pushing the sister pole away. Interaction of a microtubule end with *cut7* would produce sister pole expulsion as the microtubule is moved from molecule to molecule before detachment. The site of initial contact would be the closely apposed edges of the pole bodies, the expulsion force thereby causes the edges of the poles to tilt away from each other, allowing further microtubule-*cut7* interactions and resultant pivoting until anti-parallel symmetry is established. Microtubules of the right-hand SPB have been left out for clarity from the first two panels. *c*, SPB reorientation results from *cut7*-mediated poleward microtubule flux. Microtubules from both SPBs establish links with a third structure, X (for example kinetochore or microtubule-microtubule cross link), which is pulled towards each pole by *cut7*-directed poleward microtubule flux^{30,31}. When near to the pole any further microtubule shortening pivots the SPBs, permitting antiparallel interdigitation and resultant spindle formation. If involved in spindle elongation, centrally located *cut7* must move microtubules relative to another structure, good candidates being a spindle matrix or microtubules from the opposite pole³². If mitotic motors form a complex with two plus-end directing motor domains, one domain could interact with microtubules from either pole, simultaneously pushing the poles apart and moving the complex to the midzone. Such complexes would be efficient at sliding half spindles apart but would fail to realign parallel microtubule arrays (4a, i), rather they would 'zip' them together, creating the need for a spindle formation-specific motor.

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A yeast gene (*BEM1*) necessary for cell polarization whose product contains two SH3 domains

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CELL polarization requires that a cellular axis or cell-surface site be chosen and that the cytoskeleton be organized with respect to it. Details of the link between the cytoskeleton and the chosen axis or site are not clear¹. Cells of the yeast *Saccharomyces cerevisiae* exhibit cell polarization in two phases of their life cycle, during vegetative growth and during mating, which reflects responses to intracellular and extracellular signals, respectively. Here we describe the isolation of two mutants defective specifically in cell polarization in response to peptide mating pheromones. The mutants carry special alleles (denoted *bem1-s*) of the *BEM1* gene required for cell polarization during vegetative growth^{2,3}. Unlike other *bem1* mutants, the *bem1-s* mutants are normal for vegetative growth. Complete deletion of *BEM1* leads to the defect in polarization of vegetative cells seen in *bem1* mutants^{2,3}. The predicted

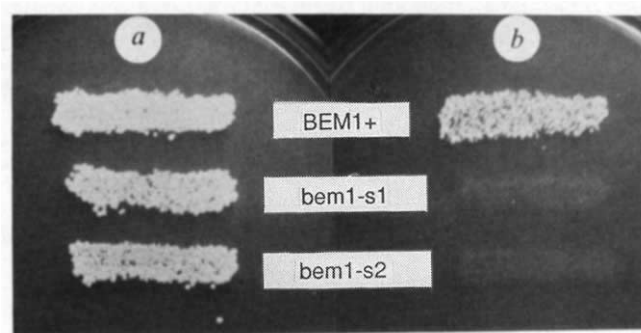


FIG. 2 Mating defect of *bem1-s* mutant cells. *a*, Mating to *BEM1*⁺ strain IH1793. *b*, mating to *bem1-s1* α strain JC107. The *a* strains are *BEM1*⁺, JC2-1B; *bem1-s1*, JC-G11; *bem1-s2*, JC-F5.

METHODS. Patches of *a* strains isogenic except at the *BEM1* locus were replica-plated to lawns of wild-type or mutant α strains on YEPD (permissive) medium and incubated at 30 °C for roughly 5 h to allow mating. These plates were then replica-plated to minimal (restrictive) medium on which only diploid cells were able to grow and incubated for a further 36 h. *MATa bem1-s* and *MATa bem1-s* strains had similar mating defects.

sequence of the *BEM1* protein (Bem1p) reveals two copies of a domain (denoted SH3) that is found in many proteins associated with the cortical cytoskeleton and which may mediate binding to actin or some other component of the cell cortex^{4,5}. The sequence of Bem1p and the properties of mutants defective in this protein indicate that it may link the cytoskeleton to morphogenetic determinants on the cell surface.

The *bem1-s* mutants were identified in a screen designed to detect nonmating mutants specifically defective in localizing cell-surface growth towards a mating partner (J.C. and I.H., manuscript in preparation; see Fig. 1 legend). In contrast to

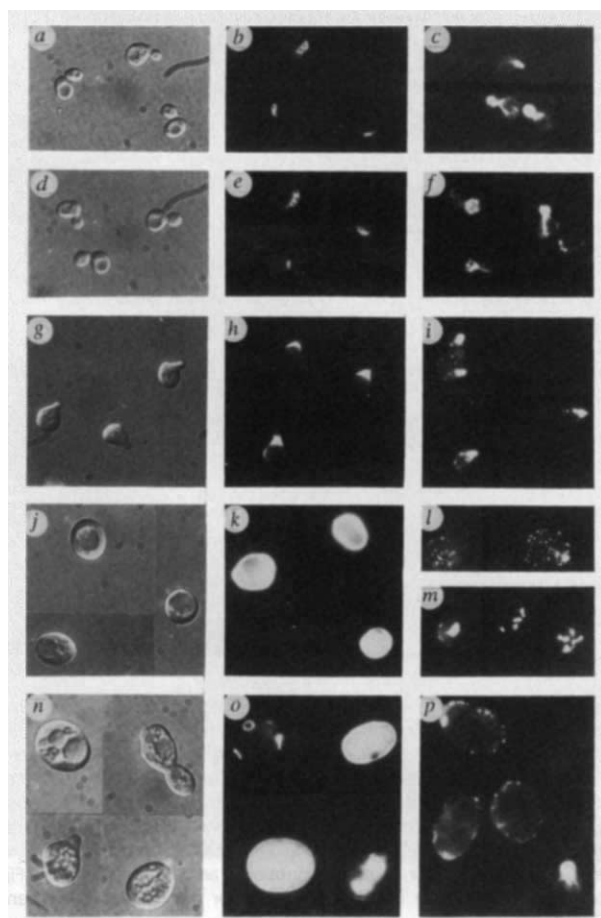


FIG. 1 Morphology (left column), chitin distribution (middle column), and actin distribution (right column) in wild-type and *bem1* mutant cells of *a* mating type. *a-c*, Wild-type strain KO2-5A; *d-f*, *bem1-s1* mutant strain JC-G11; *g-i*, wild-type strain JC2-1B treated with α -factor; *j-l* *bem1-s1* mutant strain JC-G11 treated with α -factor; *m*, *bem1-s2* mutant strain JC-F5 treated with α -factor; *n-p* *bem1*-deletion strain KO2-5C. Final magnifications are the same in all panels. Representative cells are shown.

METHODS. Mutants were isolated in a two-step screen. First, mutants were isolated that were unable to mate with strains that themselves have reduced mating ability (defective in *FAR1* (ref. 7) or in *FUS1* and *FUS2* (ref. 16)). These mutants were then examined for their morphological response to mating pheromone, which in wild-type cells induces the formation of shmoo (g). For viewing under the microscope, yeast strains were grown in YEPD medium to early log phase at 30 °C, except for the *bem1*-deletion strain, which was grown at 25 °C. Where appropriate, pheromone (α -factor; Sigma) was added to a final concentration of 10^{-6} M, and the cells were grown for a further 2–3 h. To observe morphology, live cells were viewed by differential interference contrast microscopy. Cells were fixed and stained with phalloidin to visualize actin or with Calcofluor to visualize chitin⁶. Identical results were obtained when an anti-actin antibody was used to visualize actin (data not shown). The strain deleted for *BEM1* contains the *LEU2* gene in place of the entire *BEM1* coding sequence and was constructed as follows. A fragment containing *BEM1* and several hundred base pairs of flanking DNA was cloned into a pUC vector. A portion of this plasmid was amplified by the polymerase chain reaction using divergent primers that hybridized just upstream of the *BEM1* start codon and just downstream of the stop codon. The resulting linear fragment was cleaved at a site introduced by the primers and circularized, yielding a plasmid that contained just the *BEM1* flanking regions joined by a restriction site. A selectable marker (*LEU2*) was then introduced into this site. A linear fragment containing the marker gene surrounded by the *BEM1* flanking regions was used to replace the chromosomal *BEM1* gene by the one-step gene replacement method¹⁷. Southern blotting confirmed that gene replacement had occurred by homologous recombination (data not shown).

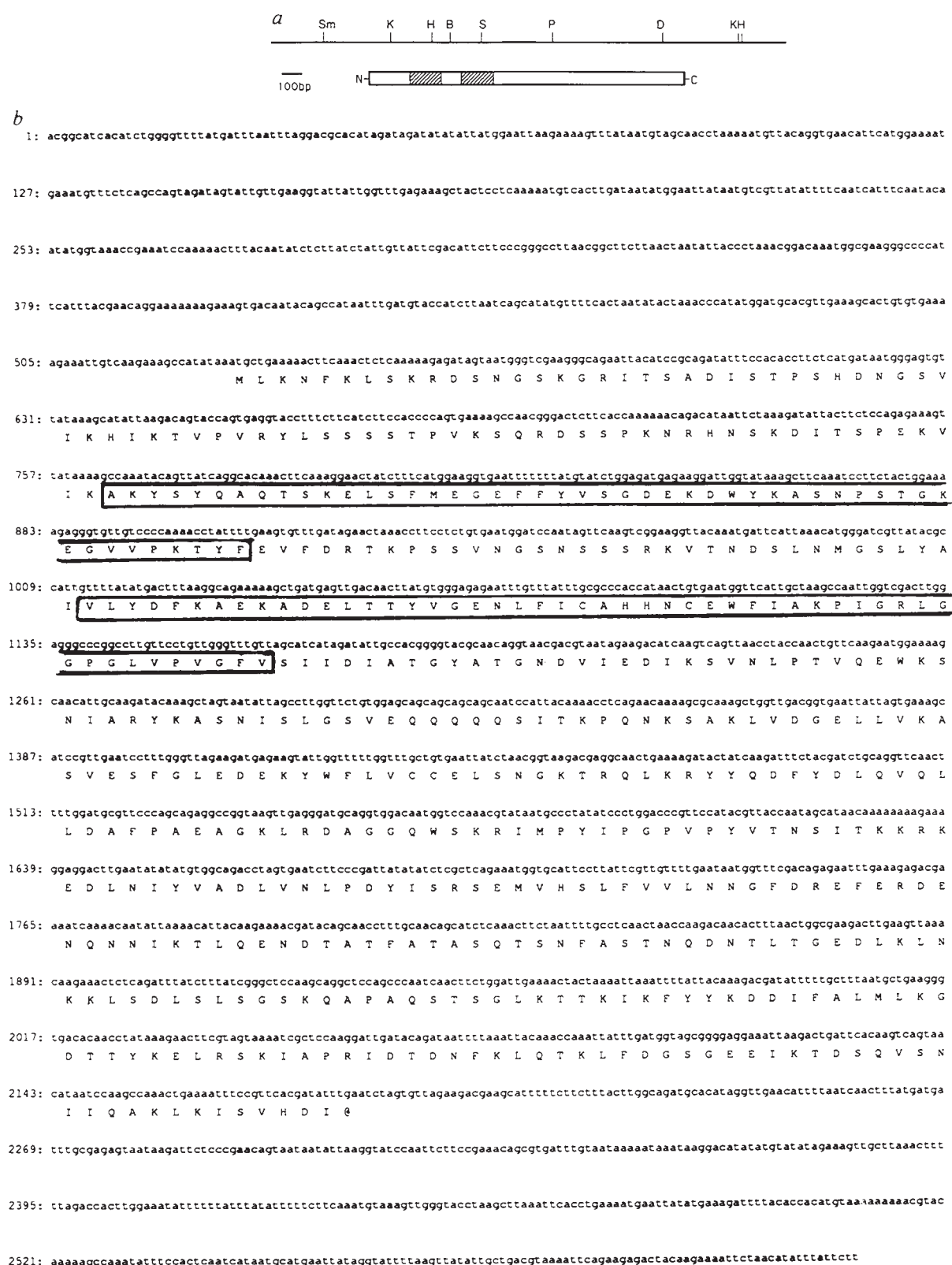


FIG. 3 *BEM1* map and sequence. **a**, Restriction map of sequenced region and location of the open reading frame. Hatched boxes indicate the positions of the SH3 domains. Restriction sites are as follows: B, *Bam*HI; D, *Dra*I; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I. **b**, Nucleotide and predicted amino-acid sequences of *BEM1*. The SH3 domains are boxed.

METHODS. *BEM1* was cloned using a genomic DNA library in a low-copy number vector (YCp50)¹⁸ by complementation of the mating defect of a *bem1-s1* strain. The complementing gene was confirmed to be the wild-type allele of the *bem1-s* mutation and not a suppressor by segregation analysis using the original *bem1-s* mutation and a mutation constructed *in vitro* in

the cloned gene which was integrated by homologous recombination (data not shown). The dideoxy chain termination method was used for sequencing M13 clones generated using restriction sites or a series of *Exo*III-generated deletions in pBluescript SK⁺ and KS⁺. The nucleotide sequences of both strands were determined independently in both laboratories and agreed exactly. The predicted protein sequence was compared with the PIR/Dayhoff database using the FASTA program¹⁹; significant homologies were detected only for proteins containing an SH3 domain (see Fig. 4). The EMBL Data Library accession number for the nucleotide sequence is X63826.



FIG. 4 Amino-acid alignment of homologous regions of Bem1p and representative SH3 domain-containing proteins. The amino acid of the first residue of each sequence is given in parentheses. Φ , Hydrophobic residues. Boxed residues, conservation with either BEM1 domain; residues that are identical are boxed and shaded. References to amino-acid sequences: *Acanthamoeba* myosin-IB²⁰, v-yes²¹, c-src²², α -spectrin²³, yeast ABP1⁵, consensus⁴.

wild-type strains, which form polarized cells called shmoo when treated with mating pheromone (Fig. 1g), two mutants were found that enlarged in a uniform manner (the Shmoo⁻ phenotype; Fig. 1j). The mutations in these strains, designated *bem1-s1* and *bem1-s2*, affect the same gene by segregation and complementation tests (data not shown). These mutants had a weak mating defect when mated to wild-type cells and a strong mating defect when mated with other *bem1-s* mutants (Fig. 2). The *bem1-s* mutants grew normally at all temperatures on rich and minimal media. Their morphologies and budding patterns were similar to those of wild-type cells (Fig. 1a, b, d, e).

To examine the polarization defect of the *bem1-s* mutants in more detail, we determined the distribution of cell-wall chitin by staining with Calcofluor and the distribution of actin by staining with rhodamine-phalloidin and by immunofluorescence using an actin-specific antibody⁶. Chitin is localized predominantly to the shmoo neck in wild-type cells exposed to pheromone (Fig. 1h). By contrast, *bem1-s* mutants deposited chitin throughout the enlarging cell surface (Fig. 1k). The actin organization in vegetatively growing *bem1-s* cells generally appeared normal: the actin patches were concentrated in the buds, and actin cables ran through the mother cells into the buds (compare Fig. 1f and c). Wild-type cells showed a similarly polarized actin distribution during shmoo formation (Fig. 1i). By contrast, the *bem1-s* cells had a delocalized actin distribution after addition of pheromone (Fig. 1l). Roughly one-third of *bem1-s2* mutant cells showed an additional, more aberrant actin distribution: 1–10 large clumps of actin per cell (Fig. 1m). The *bem1-s* mutations did not affect other aspects of pheromone response, notably cell-cycle arrest and induction of *FUS1-lacZ* (see ref. 7).

BEM1 was cloned by complementation of the mating defect of a *bem1-s1* strain (see Fig. 3 legend) and had been cloned independently by complementation of the vegetative growth defect of a *bem1-3* mutant². The nucleotide sequences of both *BEM1* isolates were identical. The predicted *BEM1* protein contains 551 amino acids (Fig. 3) with a calculated relative molecular mass of 62,000. The amino-terminal half contains two regions similar to the 'SH3 domain' (Fig. 4), a motif of 50 amino-acid residues that has been found in a variety of disparate proteins, including non-receptor tyrosine kinases, myosin-I, phospholipase C, and the yeast actin-associated protein Abp1p (ref. 5). The function of the SH3 domain is unknown, but many of the proteins containing this motif associate with the cortical cytoskeleton or localize to the plasma membrane^{4,5}.

To investigate further the role of Bem1p during vegetative growth, a strain deleted for *BEM1* was constructed. The chromosomal *BEM1* coding sequence was replaced precisely by a fragment containing *LEU2* (see legend to Fig. 1). Cells deleted for *BEM1* were able to germinate but grew slowly at room temperature or at 30 °C and not at all at 14 °C or 37 °C. Cells grown at 25 °C or 30 °C displayed heterogeneous, odd morphologies (Fig. 1n): some were either triangular or shmoo-like; others were very large and rounded and usually unbudded. Many cells were multinucleate (as seen by 4',6-diamidino-2-phenylindole staining; results not shown). Chitin deposition was delocalized (Fig. 1o) and the asymmetric actin distribution seen in wild-type cells was disrupted (Figs. 1f, p). Shift of a culture to 37 °C resulted in a population with heterogeneous terminal morphologies and an increased fraction of unbudded cells (70% versus 45% at lower temperatures). The phenotypes of the *bem1*

null mutant are similar to those of *bem1-3* and *bem1-4* mutants identified previously².

Three observations thus indicate that Bem1p has a direct role in organizing the actin cytoskeleton. First, *bem1* null mutants have a disorganized actin cytoskeleton. Second, *bem1-s2* mutants produce distinctive clumps ('bars') of actin resembling those that accumulate in certain actin⁸ or profilin⁹ mutants. Third, Bem1p contains two SH3 motifs. We propose that Bem1p has two functional domains, one (containing the SH3 domains) concerned with binding actin or some other component of the cortical cytoskeleton, the second involved with recognizing the site for cell polarization during vegetative growth or mating. In vegetative cells, the morphological landmark may be a structure that is adjacent to a site of previous budding¹⁰. The *BUD* gene products may direct the positioning of Cdc24p, Cdc42p and Bem1p to this site^{3,10,11}. During mating, a cell polarizes towards a signal from the mating partner that is probably a gradient of secreted pheromone¹². This process requires, first, an override of the vegetative bud-site selection programme and, second, establishment of polarity towards the mating partner. Erasing the bud-site selection programme may be accomplished by pheromone-induced inactivation of one or more of the *BUD* gene products. The site-recognition domain of Bem1p would then be free to interact with the new site; *bem1-s* mutants might be unable to interact with this site (but capable of interacting with the site in vegetative cells). Several observations suggest that the morphogenetic marker in mating cells might be the pheromone receptor itself. First, cells lacking the carboxy-terminal segment of the pheromone receptor have the same polarization defect as *bem1-s* mutants¹³. Second, receptors cluster at the site of pheromone-induced morphogenesis¹⁴. They seem to have a role in cell polarization that is independent of their coupled G protein and associated signalling pathway¹⁴. Spa2p is also necessary for polarization in response to mating factors¹⁵ and may interact with Bem1p or the morphogenetic determinant. By determining the location of Bem1p and molecules with which it interacts, we may begin to understand how the cytoskeleton recognizes signals on the cell surface to form a polarized cell. □

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γ -Tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation

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γ -TUBULIN is a newly identified member of the tubulin family whose sequence is highly conserved from yeast to man. This minor microtubule protein is localized to the microtubule organizing centres¹⁻³ and a mutation in the gene encoding it produces a microtubuleless mitotic arrest in the filamentous fungus *Aspergillus nidulans*^{4,5}. Here we investigate the *in vivo* function of γ -tubulin in mammalian cells using a synthetic peptide to generate a polyclonal antibody that binds to a highly conserved segment of γ -tubulin. After microinjection into cultured mammalian cells, immunofluorescence localization revealed that this antibody binds to native centrosomes at all phases of the cell cycle. In the presence of the γ -tubulin antibody, microtubules fail to regrow into cytoplasmic arrays after depolymerization induced by nocodazole or cold. Furthermore, cells injected immediately before or during mitosis fail to assemble a functional spindle. Thus *in vivo* γ -tubulin is required for microtubule nucleation throughout the mammalian cell cycle.

To prepare an anti- γ -tubulin antiserum, we synthesized a 17-amino-acid oligopeptide, EEFATEGTDRKDVFFYC, the amino-terminal 16 residues of which are conserved among all known γ -tubulin sequences (Fig. 1a). Rabbits were immunized with the peptide coupled to keyhole limpet haemocyanin. The specificity of one serum (JH46) was demonstrated by its tight binding on immunoblots to a bacterially produced mouse γ -tubulin (Fig. 1b). This serum, as well as the IgG purified from it (used in all subsequent experiments), recognized only the γ -tubulin polypeptide (relative molecular mass 50,000 (50K)) in cytoskeletal extracts of cultured mammalian cells (Fig. 1c). Binding was completely abolished by preincubation of antibody with the original γ -tubulin peptide. Immunofluorescence localization revealed γ -tubulin to be restricted to the centrosomes throughout the cell cycle^{1,2} (Fig. 1d-h), whereas immunoblotting revealed it to be exclusively in the cytoskeletal fraction (Fig. 1c).

This γ -tubulin antibody was microinjected into HeLa, mouse 3T3 and human pancreatic epithelial cells. After subsequent detergent extraction under conditions that preserve assembled microtubules⁶, the antibody bound to interphase centrosomes within 5 min of injection (Fig. 2a, b) and remained bound for at least 2 h. Although during this 2-h period the antibody did not markedly affect the array of already assembled microtubules, in 285 of 288 cells injected with γ -tubulin antibody, microtubules failed to reassemble into cytoplasmic arrays after complete disassembly induced by lowering the temperature to 4 °C for 90 min (arrowed cell in Fig. 2c, d) or by treatment with 1 μ g ml⁻¹ nocodazole for 30 min (not shown). Microtubule arrays in uninjected cells (Fig. 2c) and in all 90 cells injected with antibody prebound to the original γ -tubulin peptide rapidly reassembled a normal complement of microtubules (arrowed cell in Fig. 2e and f for example). We conclude that γ -tubulin function is necessary for the *in vivo* nucleation of interphase microtubules.

To determine whether γ -tubulin is required for *in vitro* nucleation of microtubules from centrosomes, microtubules in 3T3 or HeLa cells were disassembled with cold and the cells

were gently lysed in a solution that supports efficient microtubule assembly when supplemented with purified tubulin⁷⁻⁹. Spontaneous microtubule assembly (that is, independent of pre-formed nucleation sites) is not found under these conditions (for example, refs 7-9; see also Fig. 3c, d), but microtubule growth from centrosomes is efficient in either normal cells (not shown) or cells treated with γ -tubulin antibody prebound to the initial peptide antigen (Fig. 3a, b). However, microtubule regrowth nucleated either by interphase (Fig. 3c) or mitotic (Fig. 3d) centrosomes was completely blocked by *in vitro* incubation with the active γ -tubulin antibody. Thus, γ -tubulin function is also required for *in vitro* nucleation of microtubules from both interphase and mitotic centrosomes.

A naturally occurring microtubule disassembly/reassembly event is the cell cycle-dependent conversion of the interphase array into a mitotic spindle. At prophase, many studies (for example, refs 10-15) have demonstrated an increase by up to an order of magnitude in microtubule nucleation activity. To determine the role of γ -tubulin in spindle assembly, we injected

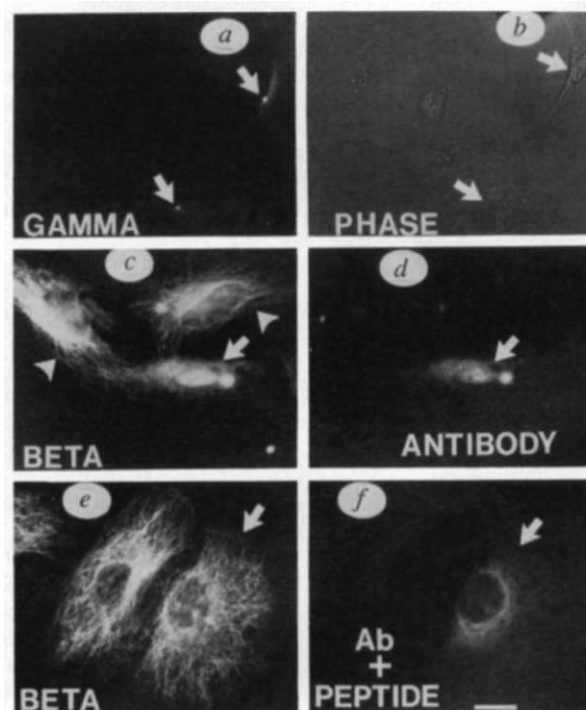


FIG. 2 Antibody against γ -tubulin blocks microtubule regrowth *in vivo* following microtubule disassembly. a, Immunofluorescent localization of microinjected γ -tubulin antibody after extracting excess antibody with detergent containing buffer (MTSB; see Fig. 1 legend); b, phase-contrast image of the lysed cells shown in a; c, Microtubule arrays regrown after cold-depolymerization of cytoplasmic microtubules in uninjected cells (arrowheads) and in a cell injected with γ -tubulin antibody (arrow); d, Immunofluorescent localization of injected γ -tubulin antibody in the same cell as shown in c; e, Microtubule arrays in a cell (arrow) that was injected with γ -tubulin antibody prebound with the original γ -tubulin peptide; f, Localization of the injected pre-bound antibody in the cell pictured in e. Scale bar, 20 μ m.

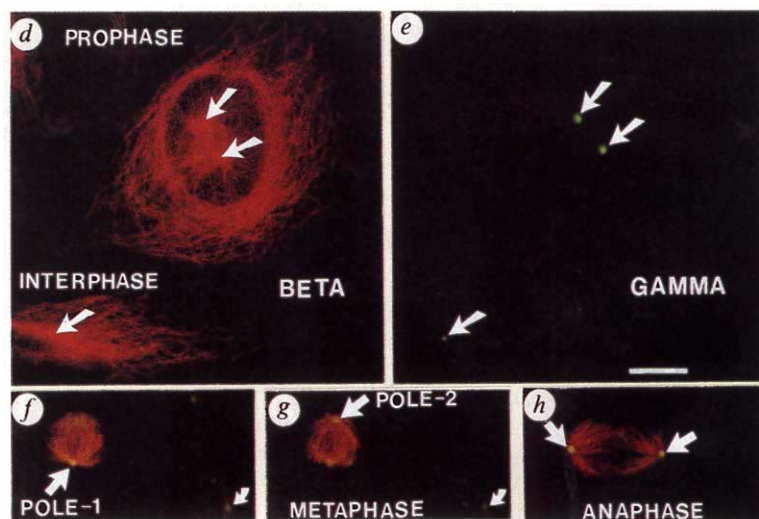
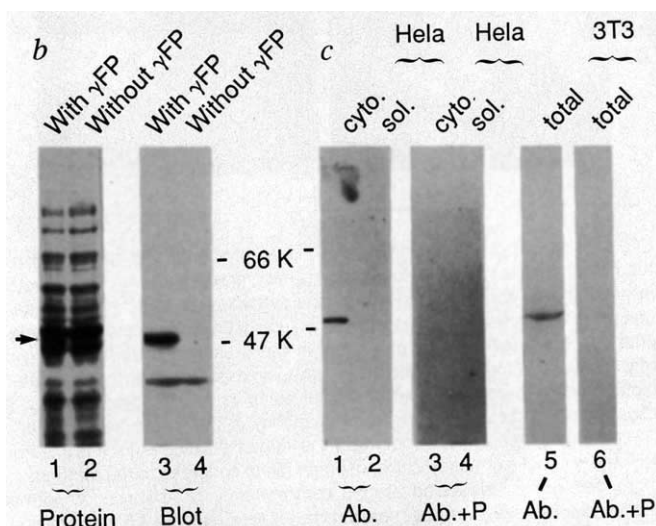
METHODS. Cells were plated at low density onto photoetched glass coverslips (Bellco) and the grid positions of cells were recorded before injection. Cells were pressure-injected (Eppendorf) into the perinuclear cytoplasm with about 0.1 μ l of γ -tubulin antibody (4 mg ml⁻¹) either untreated or preincubated with 1 mM peptide for 20 min at room temperature. Cells in a and b were then lysed in microtubule stabilizing buffer to remove excess unbound antibody, fixed and the bound antibody was detected with a fluorescein-labelled goat anti-rabbit IgG (Cooper Biomedical). Cells in c-f were incubated at 4 °C for 90 min and then allowed to recover by changing the culture medium and incubating at 37 °C. After 30 min, cells were fixed in cold methanol and stained with mouse anti- β -tubulin antibody and a mixture of fluoresceinated goat anti-rabbit antibody and rhodaminated goat anti-mouse antibodies. This procedure allowed the visualization of the injected antibody (d, f) in one channel and the microtubule arrays in the other (c, e).

the γ -tubulin antibody into cells that had been synchronized by double thymidine block and then released. Although injected cells entered mitosis on schedule (as judged by chromosome condensation and nuclear envelope breakdown), in 93 of 146 cells injected with γ -tubulin antibody an aberrant spindle with a diminished number of microtubules was observed (for example, arrowed cell in Fig. 4a) and the remaining 53 cells showed a near-complete absence of spindle microtubules (for example, arrowed cell in Fig. 4d). In all cells, chromosomes failed to congress to the midplate and were often clustered adjacent to the duplicated centrosomes (Fig. 4a, b), presumably

tethered there by the few, short microtubules that remained. When the antibody was injected after chromosome alignment, the chromosomes remained at the midzone even though spindle microtubules diminished in numbers and lengths (Fig. 4d, e). By sharp contrast, spindle microtubules were unaffected in all 53 cells injected with preimmune antibody and in 63 of 67 cells injected with immune antibody prebound with peptide. (Similar high concentration injections of six independent α - or β -tubulin antibodies before or during mitosis have no effect on the morphogenesis of normal mitotic spindles¹⁶, although high concentrations of a monoclonal antibody against tyrosylated

<i>a</i>	38	53
Peptide	EEFATEGTDRKDVFFY-C	
Human	EEFATEGTDRKDVFFY	
Mouse	EEFATEGTDRKDVFFY	
<i>Drosophila</i>	EDFANDGLDRKDVFFY	
<i>Aspergillus</i>	EEFATEGGDRKDVFFY	
Yeast (<i>S. pombe</i>)	ESFATEGVDRKDVFFY	
α -tubulin	SDKTIGGGDDSFNTFF	
β -tubulin	G ² SD ¹ QL ² RISVXYNE	

FIG. 1 A γ -tubulin antibody generated against a peptide conserved among γ -tubulins from yeast to man. *a*, Sequence of the γ -tubulin peptide used to elicit γ -tubulin antibodies aligned with the corresponding domains (γ -tubulin residues 38–53) from human¹, mouse, *Drosophila*², *Aspergillus*⁴ and *Schizosaccharomyces pombe*^{2,3} γ -tubulins. The mouse sequence was determined from a complementary DNA clone isolated by hybridization with a *Drosophila* γ -tubulin cDNA. Note the divergence of the γ -tubulin peptide sequence from the corresponding sequences from mammalian α -tubulin (represented by human genes $\beta\alpha 1$ and $\kappa\alpha 1$ (ref. 18), a porcine sequence¹⁹, a rat α -tubulin²⁰, and two Chinese hamster α -tubulins²¹) and the consensus sequences of mammalian β -tubulin isotypes expressed in fibroblasts^{22,23}. *b*, *c*, Specificity of the γ -tubulin polyclonal antibody determined by immunoblotting. *b*, Coomassie blue-stained gels of bacterial lysates containing (lane 1) or lacking (lane 2) a bacterially derived γ -tubulin fusion protein; lanes 3, 4, immunoblots using the γ -tubulin polyclonal antiserum of samples in lanes 1 and 2. *c*, Immunoblots of cytoskeletal (lane 1) (cyto) and soluble (lane 2) (sol) fractions of HeLa cells using the rabbit polyclonal anti γ -tubulin antibody (Ab) (40 $\mu\text{g ml}^{-1}$); lanes 3, 4, immunoblots as in lanes 1 and 2 except the antibody was bound in the presence of 0.5 mM competing γ -tubulin peptide (P); lane 5, immunoblot of mouse 3T3 whole-cell extract using 40 $\mu\text{g ml}^{-1}$ γ -tubulin antibody; lane 6, immunoblot as in lane 5, except that the antibody binding was in the presence of 0.5 mM competing γ -tubulin peptide. *d–h*, Immunofluorescence localization of γ -tubulin during the mammalian cell cycle: *d*, microtubule arrays visualized with a β -tubulin antibody in an interphase cell and in a cell that is in early prophase; *e*, localization of γ -tubulin in the same cells shown in *d* (arrows point to the centrosomes); *f–h*, double immunofluorescence localization of β -tubulin (red) and γ -tubulin (green) in two focal planes of a metaphase spindle (*f*, *g*) and an anaphase spindle (*h*). Scale bar, 20 μm . **METHODS.** For antibody generation, the γ -tubulin peptide was coupled through the C-terminal cysteine to maleimide-activated keyhole limpet haemocyanin (Pierce, Rockford). Following emulsification with complete Freund's adjuvant (2 mg antigen in 1 ml suspension), the immunogen was injected subcutaneously into rabbits. Booster injections were given after two weeks and blood drawn after the third week. Sera were screened by immunoblotting against a bacterially produced protein containing 13 amino acids of β -galactosidase linked to residues 34–451 of mouse γ -tubulin. IgG was purified from whole antiserum by chromatography on a protein A/G-agarose column (Pierce). For preparation of 3T3 and HeLa cell extracts, cells were cultured in DMEM with 10% fetal calf serum, 5% CO_2 in a 37 $^\circ\text{C}$ humidified incubator. After removal of the medium, soluble contents



were extracted in microtubule stabilizing buffer (MTSB; 0.1 M 2-N-morpholinoethane sulphonic acid, pH 6.75, 1 mM MgSO_4 , 2 mM EGTA, 0.1 mM EDTA, 0.1 M GTP, 4 M glycerol, 1% Triton X-100) as described earlier²⁴. After 5 min, residual cytoskeletal proteins were solubilized in 0.1 M Tris, pH 6.8, 1% SDS. Total cell extracts were prepared by solubilizing cells directly in 0.1 M Tris, pH 6.8, 1% SDS. Protein concentrations were determined by bicinchoninic acid assay²⁵. Both fractions were brought to identical volumes and equal proportions of each were then analysed on SDS-polyacrylamide gels followed by immunoblotting with γ -tubulin antibody. For immunofluorescence localizations, cells on glass coverslips were lysed in microtubule stabilizing buffer⁶ and fixed by plunging into methanol at -20°C for 5 min. Coverslips were rehydrated in PBS and stained with the γ -tubulin antibody (40 $\mu\text{g ml}^{-1}$) or a β -tubulin monoclonal antibody (Amersham) for 1 h at room temperature. They were then rinsed with PBS and either a fluorescein labelled anti-rabbit IgG antibody and a rhodamine-labelled anti-mouse IgG antibody (Cooper Biomedical) was applied for 45 min at room temperature. The coverslips were rinsed with PBS and mounted in Aquamount (Lerner Laboratories, New York). The cells were examined on a Zeiss Axiovert microscope with epifluorescence optics.

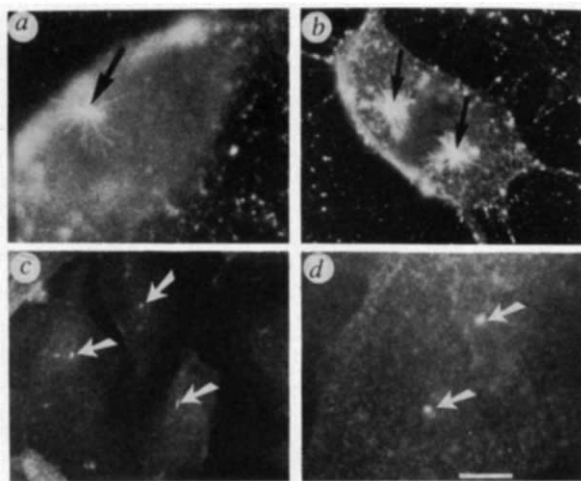
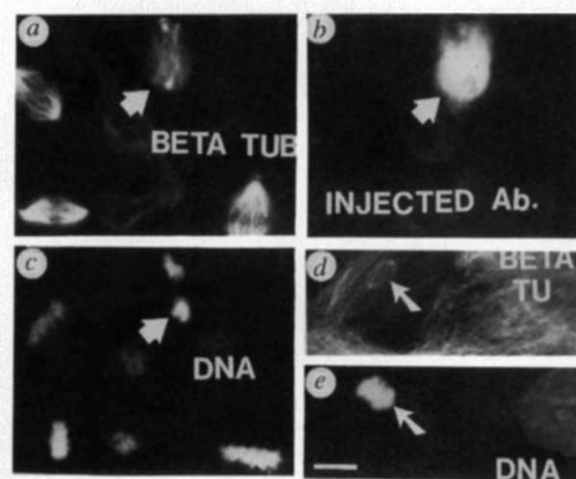


FIG. 3 Antibody against γ -tubulin blocks nucleation of microtubule growth from centrosomes in permeabilized cells. *a*, Immunofluorescence micrograph showing polymerization of chicken brain tubulin nucleated *in vitro* from the centrosomes of permeabilized interphase (*a*, *c*) or metaphase cell (*b*, *d*) in the presence of either the antibody pre-bound to the original γ -tubulin peptide (*a*, *b*) or untreated γ -tubulin antibody (*c*, *d*). Metaphase cells were evident in phase optics by their aligned chromosomes at the midzone, the relative position of spindle poles, and the absence of the nucleus. Scale bar, 20 μ m.

METHODS. Cells (NIH3T3) grown on glass cover slips were incubated at 0–4 °C for 90 min and then gently lysed following established methods^{6–8} in PEM buffer (80 mM PIPES, pH 6.9, 1 mM EGTA, 0.5 mM MgSO₄, 1 mM GTP, and 0.05% Triton X-100). Lysed cells were then incubated with γ -tubulin antibody (2 mg ml⁻¹ in PEM) for 20 min at 25 °C in presence or the absence of 0.5 mM peptide antigen. Cells were then incubated for 20 min at 37 °C with 2.2 mg ml⁻¹ purified chicken brain tubulin, washed briefly with microtubule stabilizing buffer and fixed in cold methanol at –20 °C. The newly formed microtubules were visualized by their immunofluorescent staining using a mouse monoclonal β -tubulin antibody.

FIG. 4 γ -tubulin antibody disrupts the assembly of mitotic spindle microtubules. *a*, Indirect immunofluorescence micrograph showing the arrangement of spindle microtubules in four cells synchronized to be in metaphase, one of which (arrow) was injected with γ -tubulin antibody immediately after envelope breakdown during prophase. In the presence of antibody only a few short and disoriented microtubules remain assembled at the duplicated centrosomes, in contrast to the normal spindles in the adjacent cells. *b*, Localization of the injected γ -tubulin antibody in the same field as in *a*. *c*, DAPI (diaminophenylindole) staining of the same field of cells. In cells injected with the γ -tubulin antibody, chromosomes fail to congress to the metaphase plate and remain clustered around centrosomes. *d*, Absence of normal microtubule arrays in a degenerated metaphase spindle 15 min after γ -tubulin antibody was injected (arrow). *e*, DAPI staining of the same field of cells as in *d*. Chromosomes remain at the metaphase plate despite the loss of many spindle microtubules. Scale bar, 20 μ m.

METHODS. Cells (human CF-PAC, cystic fibrosis pancreatic cancer) were cultured on photoetched glass coverslips, synchronized by double thymidine block, and then released for 4–6 h. Selected cells were microinjected with about 0.1 μ l of 4 mg ml⁻¹ γ -tubulin antibody (cell marked by the arrow) or the same amount of pre-immune antibody isolated from pre-immune serum by binding to protein A/G agarose, or 4 mg ml⁻¹ γ -tubulin antibody preincubated with 1 mM peptide (for 20 min at room temperature) at or before prophase (*a*) or during metaphase (*d*). Prophase and metaphase cells were identified by observing DNA condensation, nuclear envelope breakdown, and the presence of a metaphase plate under phase optics. Cells were fixed 15 min later and stained post-injection with an anti- β -tubulin mouse mono-



clonal antibody (Amersham) for 60 min followed by RITC-goat anti-mouse IgG, FITC-goat anti-rabbit IgG and DAPI. Cells were washed in PBS, mounted in Aquapmount (Lerner), and photographed using a Zeiss Axiocvert microscope with epifluorescence optics.

α -tubulin induces perinuclear bundles of microtubules that interfere with mitosis¹⁷).

That γ -tubulin may be involved in the nucleation of microtubules was first suggested by inferences drawn from combining genetic⁵ and immunocytochemical observations^{1–3,5}. Mutant alleles in the *mipA* gene (encoding γ -tubulin in *Aspergillus*) resulted in microtubuleless mitotic arrest⁵, whereas immunofluorescent localization revealed the presence of γ -tubulin in fungal spindle pole bodies^{3,5} and at mammalian and amphibian centrosomes^{1,2}. In this context, the present evidence provides a direct *in vivo* link for γ -tubulin function in microtubule nucleation during interphase and mitosis. Three general possibilities arise as to how antibody binding blocks nucleation. First, the antibody may sterically occlude specific interactions of γ -tubulin with other tubulin subunits. This view is also supported by the observation that some γ -tubulin mutations can suppress specific β -tubulin mutations⁴, highly suggestive of a direct interaction. A second possibility is that the antibody disrupts γ -tubulin interactions with other centrosomal components. This seems less likely because after antibody injection all γ -tubulin remains centrosomal even though microtubule arrays fail to regrow; hence, at least some interactions that bind γ -tubulin to centrosomal components are not affected. The formal possibility that the binding of γ -tubulin antibody sterically prevents microtubule nucleation from a non- γ -tubulin

component cannot be excluded, but in light of γ -tubulin's localization, conservation and proven essential role in lower eukaryotes, it is likely to have a central, if more indirect, role in microtubule nucleation. Finally, γ -tubulin antibody binding may directly inactivate some functionally important enzymatic activity of γ -tubulin (such as a putative GTPase activity analogous to that found in the tubulin heterodimer of α - and β -subunits). Whatever the mechanism, it is clear that at least one function of γ -tubulin is an intrinsic involvement in centrosome-dependent nucleation of microtubule growth at all phases of the cell cycle. □

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Assessment of protein models with three-dimensional profiles

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AS methods for determining protein three-dimensional (3D) structure develop, a continuing problem is how to verify that the final protein model is correct. The revision of several protein models to correct errors^{1–6} has prompted the development of new criteria for judging the validity of X-ray^{7–9} and NMR^{10,11} structures, as well as the formation of energetic^{12–14} and empirical methods^{15,16} to evaluate the correctness of protein models. The challenge is to distinguish between a mistraced or wrongly folded model, and one that is basically correct, but not adequately refined. We show that an effective test of the accuracy of a 3D protein model is a comparison of the model to its own amino-acid sequence, using a 3D profile¹⁶, computed from the atomic coordinates of the structure. 3D profiles of correct protein structures match their own sequences with high scores. In contrast, 3D profiles for protein models known to be wrong score poorly. An incorrectly modelled segment in an otherwise correct structure can be identified by examining the profile score in a moving-window scan. The accuracy of a protein model can be assessed by its 3D profile, regardless of whether the model has been derived by X-ray, NMR or computational procedures.

The method, outlined in Fig. 1, measures the compatibility of a protein model with its sequence, using a 3D profile. Each residue position in the 3D model is characterized by its environment¹⁷ and is represented by a row of 20 numbers in the profile. These numbers are the statistical preferences (called 3D–1D scores) of each of the 20 amino acids for this environment. Environments of residues are defined by three parameters: the area of the residue that is buried; the fraction of side-chain area that is covered by polar atoms (O and N); and the local secondary structure. The 3D profile score S for the compatibility of the sequence with the model is the sum, over all residue positions, of the 3D–1D scores for the amino-acid sequence of the protein. As described below, the compatibility of segments of the sequence with their 3D structures can be assessed by plotting, against sequence number, the average 3D–1D score in a window of 21 residues.

For 3D protein models known to be correct, the 3D profile score S for the amino-acid sequence of the model is high. This is illustrated in Fig. 2 where the scores of all very well determined structures are indicated by large dots. By contrast, the profile score S for the compatibility of a wrong 3D protein model with

its sequence is often low, as shown by the seven squares in Fig. 2 and discussed below. The 3D profile scores for four models that are largely misfolded all fall below the dotted line. Three other faulty structures that contain some correct as well as incorrect segments have 3D profile scores that fall between the two lines.

The profile score of a model depends on its size and its validity. Profile scores of correct models increase with the length of the protein, simply because more positive residue preferences are added into the sum. Small proteins may also score somewhat less than large ones because they have a smaller fraction of internal residues and hence give less informative profiles. Structures of the same length determined by X-ray and NMR tend to have comparable scores. The scores for computationally based models vary, depending on their accuracy. The deliberately misfolded models of Novotny *et al.*¹² have low scores because the environments of residues in the incorrect 3D structures are not compatible with the residues in the corresponding positions of the sequence. By contrast, models based on structures having

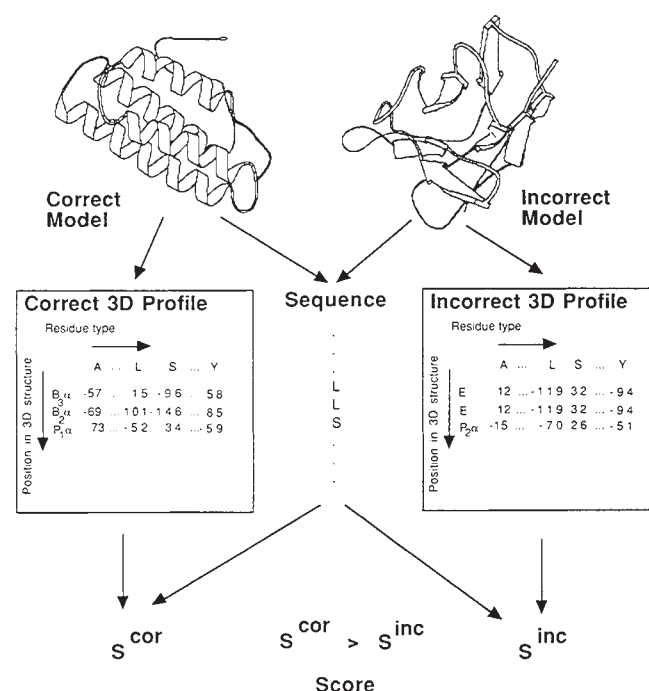


FIG. 1 The use of 3D profiles to verify protein models, illustrated with the correct and misfolded structures of haemerythrin, both having 113 residues. The model on the left is the X-ray-derived structure²⁹. A 3D profile calculated from its coordinates matches the sequence of haemerythrin with a score $S^{cor}=38$. The right hand model is the misfolded haemerythrin model of Novotny *et al.*¹². A 3D profile calculated from it matches its sequence poorly, with score $S^{inc}=15$. The actual profile consists of 113 rows (one for each position of the folded protein). In this schematic example only 3 rows are shown, those for positions 33 (where the residue is L), 34 (L) and 35 (S) (single-letter amino-acid code). The first column of the profile gives the environmental class of the position, computed from the coordinates of the model¹⁷, and the next 20 columns give the amino-acid preferences (called 3D–1D scores) at that position. In this schematic example, there are only four columns of 3D–1D scores shown, those for residues, A, L, S and Y. In the correct profile on the left, position 33 is computed to be in the buried polar α -helical class ($B_3\alpha$); positions 34 and 35 are computed to be in the buried moderately polar α -helical class ($B_2\alpha$) and the partially buried α -helical polar class, $P_1\alpha$, respectively. The scores for the residues L, L and S for these three positions are 15, 101 and 34. The profile for the misfolded model assigns positions 33, 34 and 35 to the environmental classes E, E and $P_2\alpha$, giving 3D–1D scores for the residues L, L and S of –119, –119 and 26, respectively. That is, in the incorrect structure, the leucine residues are exposed, giving low 3D–1D scores and leading to a summed total score S^{inc} which is much smaller than S^{cor} when all other 3D–1D scores are summed along with the three shown here.

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TABLE 1 3D profile scores of correct and incorrect/initial protein models with their own amino-acid sequences

Model	Correct	Incorrect/initial	Length (residues)
Rubisco small subunit	55.0 (3RUB)*†	15.1 (ref. 23)	123
Ferredoxin of <i>A. vinelandii</i>	45.9 (4FD1)* (ref. 2)	10.8 (2FD1) (ref. 24)	106
Ig κ V-region	47.0‡	6.9‡	113
Haemerythrin	37.9‡	14.9‡	113
<i>ras</i> p21	79.8(2P21)* (ref. 5)	50.5 (ref. 25)	177
EcoRI	116.5 (ref. 6)	89.3 (ref. 26)	261
HIV protease	53.9(3HVP)*	33.7 (ref. 27)	97

Notice that correct models match their own sequences with higher scores than do incorrect models. Scores of incorrect/initial models are shown in Fig. 2 by squares.

* Brookhaven Protein Data Bank codes²⁸.

† P. Curmi *et al.*, manuscript in preparation.

‡ Energy minimized models, described in ref. 12.

closely related amino-acid sequences, such as those for cyclic-AMP-dependent protein kinase (Brookhaven Protein Data Bank model 2APK)¹⁸, insulin-like growth factor (1GFI, 2GFI)¹⁹, apolipoprotein D (1APD)²⁰, and protein C inhibitor (2PAI)²¹, have high scores comparable to many X-ray and NMR models. This contrast suggests that 3D profiles can distinguish between correct and misfolded models, however developed, and will be useful in assessing computational models and *de novo* protein designs. If this is so, then none of the three δ haemolysin models²² deposited with the Brookhaven Protein Data Bank (1DHL; the lowest circles of Fig. 2) is correct.

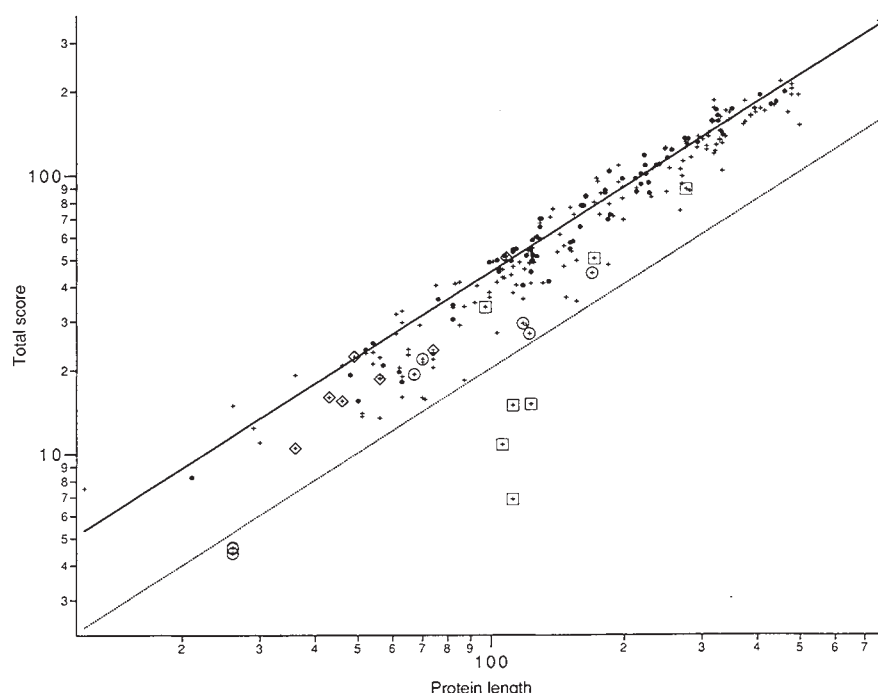
Seven examples of profiles from misfolded models are given in Table 1 and illustrated by profile window plots in Fig. 3. One example is that of the small subunit of Rubisco, which was traced essentially backwards from a poor electron-density map²³. The profile calculated from this mistraced model gives a score of only 15 when matched to the sequence of the small subunit of Rubisco. This score is well below the value of about 58 expected for a correct structure of this length (123 residues). In

fact, the profile for the correct model of the small subunit of tobacco Rubisco (P. Curmi, H. Schreuder, D. Cuscio, R. Sweet and D.E., manuscript in preparation) matches its sequence with a score of 55. Also, when the score for the mistraced small subunit is plotted as a function of the sequence, as in the profile window plot of Fig. 3, the average score is often below the value of 0.1 and dips below zero at two points. As a control, 71 well-refined X-ray structures (having *R*-factors below 0.20, and resolutions of 2 Å or finer) were also examined by profile window plots. None of these profile window plots dipped below zero. Other faulty models whose profile window plots are shown in Fig. 3 include the mistraced ferredoxin from *Azotobacter vinelandii*²⁴, the deliberately misfolded protein models of Novotny *et al.*¹², the initial model of *ras* p21 protein, which had two β strands interchanged²⁵, the initial model of *EcoRI* endonuclease²⁶ which had several improperly built segments, and the initial model of the human immunodeficiency virus (HIV) protease²⁷ which was improperly interpreted at the C terminus. All of the incorrect models have profile window plots that are largely or completely below the plots for the correct models, and in all cases the plots for the incorrect models signal a problem by approaching or falling below a score of zero.

Of the examples of Fig. 3, *ras* p21 presents the most interesting case of profile window plots. In the initial model of *ras* p21 protein²⁵, β strands 1 and 3 had been interchanged along with associated loops, corresponding to changes in the vicinity of residues 1–11 and 46–65. The window plot reveals the problem in the region 46–65, but because of the averaging procedure, misses the problem at the N terminus. Also the initial model contained a one-residue misregistration between residues 98–107, and this problem is reflected by the dip in the average score in this region. Ramachandran diagrams are not so effective in revealing the faulty segments: in the initial structure, 33% of all non-glycine residues lie outside the allowed regions, but only 32% of non-glycine residues in the faulty segments lie outside. Similarly for the deliberately misfolded V-region domain¹² of Table 1 and Fig. 3, the Ramachandran diagram appears normal, even though the structure is completely wrong.

Our experience with profile assessment of faulty models can be summarized as follows. Models that are largely incorrect can often be detected by low overall profile scores (Table 1; Fig. 2).

FIG. 2 3D profile scores (+) for protein coordinate sets in the Brookhaven protein data bank²⁸, as a function of sequence length, on a log-log scale. All + signs that are not enclosed represent X-ray determinations. ●, Scores for highly refined X-ray determinations; these are structures determined at resolutions of at least 2 Å and with *R*-factors <20%. The heavy line is fit by least squares to these highly refined structures. ◇, Scores for NMR structures; ⊕, scores for computationally determined models; □, misfolded structures: these correspond to the entries of Table 1 and also the lower curves of Fig. 3. Notice that severely misfolded structures and some computational models have scores below $0.45 \times S^{\text{calc}}$ shown by the dotted line. Environmental classes for 3D profiles of oligomeric proteins were generally computed from oligomeric structures, rather than protomers. The difference is that the accessible surface areas of residues positioned at interfaces are greater for the protomers, producing a poorer fit of the profile to the sequence. This is insignificant for large structures, but important for small structures. As an extreme case, the profile for the 12-residue designed protein α 1 (ref. 30) (the + at the left of the figure) matches its sequence only when the molecule is surrounded by its neighbours as in the crystal structure. Profile scores are plotted for coordinate sets in the January 1991 release (most recent entry when multiple entries are present) plus NMR coordinates in the April 1991 release.



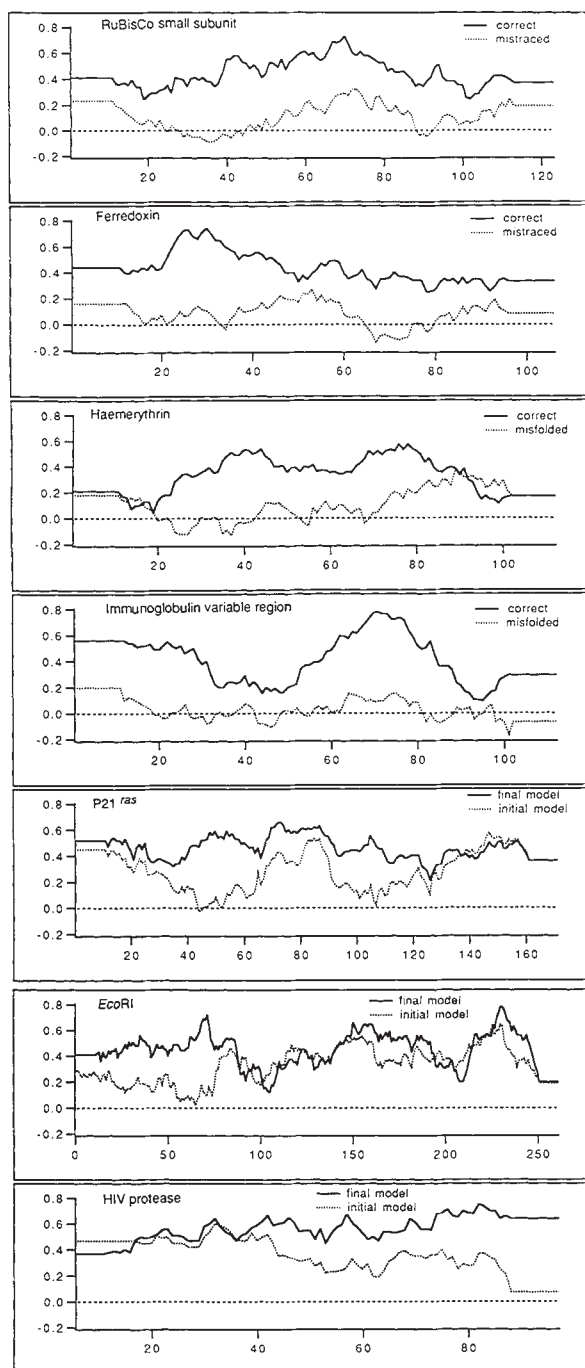


FIG. 3 Profile window plots for various incorrect and partially incorrect protein models, compared with window plots for their correct counterparts. The vertical axis gives the average 3D-1D score for residues in a 21-residue sliding window, the centre of which is at the sequence position indicated by the horizontal axis. Scores for the first 9 and the final 9 sequence positions have no meaning. A window length of 21 residues strikes a useful balance between smoothing fluctuations and localizing the error.

Models that contain a small number of improperly built segments can be detected by low scoring regions in a profile window plot (Fig. 3). But not all faulty regions are always evident directly from the profile, particularly if the misbuilt regions are at the termini, where they are obscured by the windowing procedure. In practice, however, once a profile reveals any improperly built region in a protein, reinspection of the model may uncover other problems. In short, Figs 2 and 3 suggest that a correct protein model can be verified by the compatibility of its 3D profile with its sequence, and that a misfolded model can often be detected by its incompatibility.

An advantage of using 3D profiles for testing models is that the profiles have not themselves been used in the determination of the structure. In X-ray analysis, the final model is generally obtained by systematic variation of the model, forced by minimization of a function closely related to the *R*-factor. The *R*-factor is then also used as a criterion for assessing the accuracy of the model. Thus in essence, the model is judged by a test that is inherent in its formulation. Similarly, in construction of models by molecular mechanics and molecular dynamics, the final model is arrived at by minimization of energy. If no experimental structure is available for comparison, energetic analysis enters into both the determination and the evaluation of the structure. By contrast, the 3D profile method for model evaluation proposed here relies only on a comparison of the model to its own amino-acid sequence. Thus the present method not only seems effective in assessing protein models, but it also avoids the circularity that is to some extent inherent in the common methods of evaluation of 3D protein models. □

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Electrospray LC/MS — can it be used to determine lower molecular weight molecules?

Robert D. Voyksner

A brief description of electrospray theory and operation is presented with emphasis on the determination of lower molecular weight molecules.

Mass spectrometry (MS) is an important tool for determining environmentally and biologically relevant compounds. With a need for increased knowledge of non-volatile and thermally unstable polar organics in complex mixtures, the development of liquid chromatographic (LC) separation techniques coupled with soft desorption ionization techniques with MS have been important breakthroughs. One of the most promising LC/MS techniques using a soft ionization technique is electrospray.

Electrospray operation

The electrospray technique, pioneered by Dole¹ and combined with mass spectrometry by Fenn^{2,3}, has recently revolutionized mass spectrometry with its ability to ionize high molecular weight molecules at efficiencies that permit femtomole detection limits. The electrospray process (see Fig. 1) can be characterized by four discrete steps: (1) nebulization and charging of the LC effluent, (2) desolvation, (3) ionization, and (4) extraction of ions into the MS. The technique is limited to LC flow rates of 1–10 $\mu\text{l min}^{-1}$, which can be achieved either by using capillary LC or splitting the eluent from a conventional-sized 2- or 4.6-mm i.d. LC column. The initial step of the process, nebulizing and charging, occurs when the LC eluent is introduced into a cylindrical electrode through a stainless steel hypodermic needle, which is at ground potential (Fig. 1). A high electrical field, 3–4 kV, on the cylindrical electrode charges the surface of the emerging liquid forming a fine spray (Taylor cone) of charged droplets. Driven by electric fields, the droplets migrate to the capillary through a counter flow of heated nitrogen, which helps desolvate the droplets as well as carry away uncharged material. This nitrogen prevents fouling of the capillary, the skimmers and the mass analyser. The ionization of the analyte in the desolvating droplets is by an ion evaporation process that does not require sample volatility.

Supersonic free gas transport then carries analyte ions arriving at the vicinity of the capillary inlet to the first vacuum chamber. A portion of the free jet flow passes further through a skimmer into a second vacuum chamber for mass analysis.

Ionization

The key feature of electrospray is the formation of an ion through the ion evaporation process. The charged droplets formed by the high-voltage electrode at the hypodermic needle desolvate in the nitrogen to a point where repulsive Coulombic forces exceed the droplet cohesive forces leading to ion evaporation. The two mechanisms that are cited for ion

electrospray is a soft ionization process yielding only molecular ions.

Another important feature of the ionization process is the ability to form multiply-charged ions, depending on the acid/base chemistry and the hydration energy of the molecule^{8–10}. Such ions permit the analysis of higher molecular weight compounds using existing, conventional mass analysers (for example, ions above m/z 2,000 are seldom observed). This effective increase in the mass range is the result of multiple charge placement (increasing z) to give a m/z ratio in the range of 0–2,000 Daltons. The mass spectrometrists can now enter the world of the biologist or polymer chemist, providing molecular weight information on peptides, proteins, oligonucleotides, glycoproteins, and polymers in excess of 100 kD (refs 9–14).

Lower molecular weights

In the rush to solve the large molecule problems of biochemistry and biology, using the capabilities of electrospray LC/MS to examine small molecules of environmental or pharmaceutical interest has received limited attention. However, the same advantages of femtomolar sensitivity observed for the detection of proteins should also be realized for the analysis of low molecular weight molecules. On the other hand, the soft ionization process will preclude the formation of structurally relevant fragment ions, which can be a major weakness in attempts to identify or confirm the presence of a particular drug or environmental contaminant. Although more costly tandem MS techniques can be used to overcome this deficiency, it is also possible to record collisional activation decomposition (CAD) spectra generated in the electrospray transport region between the capillary and first skimmer by controlling the potential difference between these two elements (CAD region, Fig. 1). At increased potential differences, all ions are accelerated in the high pressure supersonic expansion region resulting in collisional activation. As the potential dif-

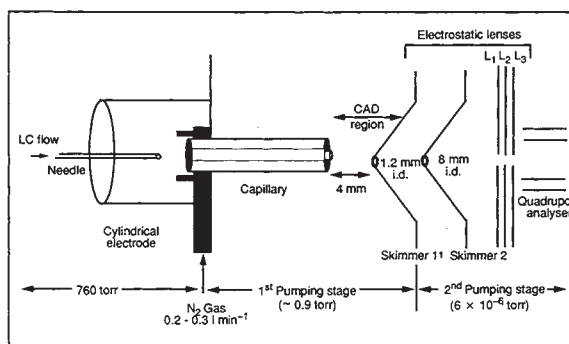


FIG. 1 Electrospray-mass spectrometry interface.

formation from these charged droplets are direct field evaporation of ions and droplet fusion at the Rayleigh limit to form charged residues. It has been postulated that micro-sized droplets generated in electrospray undergo a cascade of fusion processes yielding smaller and smaller droplets until the electric field at the droplet surface is sufficient for direct ion evaporation^{4,5}. It appears that best performance is achieved for analytes that carry a net charge in solution. These analytes can form ions in the gas phase in a variety of solvents indicating the broad applicability of the technique, particularly when the pH of the solution can be controlled. Furthermore, the ionization process appears very efficient as it does not depend on statistical charging of droplets as in thermospray^{6,7}. Finally, the ion evaporation-ionization in

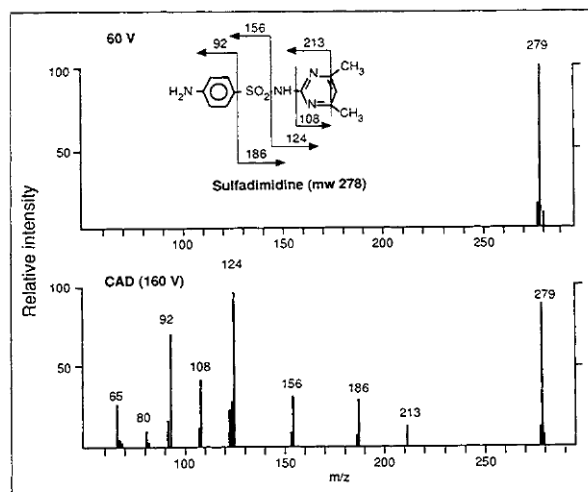


FIG. 2 Electrospray CAD (collisional activation decomposition) spectra of sulfadimidine. Top, mass spectrum obtained at a capillary-skimmer potential difference of 60 V. Bottom, mass spectrum obtained under CAD conditions with a capillary-skimmer potential difference of 160 V.

ference is increased, enough internal energy can be imparted to the molecules to produce the multiple bond cleavage necessary for structure elucidation. This method of collisional activation has been shown to introduce over 16 eV of internal energy into the molecule, yielding extensive fragmentation with less than 20 per cent loss in ion current¹⁵. These internal energies and ion yields can be significantly higher than those measured from tandem MS¹⁵. The capability to control the CAD energy by increasing the potential difference between capillary and skimmer can change the simple, single-ion spectrum into an information-rich spectrum almost instantly. For example, compare the spectra for sulfadimidine recorded at a potential difference of 60 V and 160 V (see Fig. 2). At a capillary-skimmer difference of 160 V, sulfadimidine exhibits eight significant fragment ions with less than 10 per cent loss in total ion current. Furthermore, the CAD spectra generated in the electrospray transport region are qualitatively similar to the spectra recorded on a tandem MS instrument.

The effectiveness of electrospray LC/MS for pharmaceutical or environmental applications has been demonstrated for the analysis of a variety of toxins, β -lactam antibiotics, aminoglycosides, nucleoside bases, sulfamides, steroids, glycerides, carbamate pesticides, triazine herbicides, azo dyes and other selected drugs and polar organic molecules¹⁵⁻²¹. Electrospray MS full-scan detection limits of 1-10 pg have been achieved for many low molecular weight pesticides and drugs such as aldicarb and penicillin G (ref. 15). The use of CAD also provides the specificity for identification or target residue confirmation.

In conclusion, electrospray appears to have a superior combination of sensitivity and specificity (CAD in source) for the determination of picogram quantities of non-volatile low molecular weight molecules. However, electrospray LC/MS is not yet routine, and some work in optimization and development of micro tech-

niques for sample handling and separation (microbore LC) is needed in order to achieve the full capabilities of the technique. It appears that electrospray will play a rapidly increasing role in the determination of non-volatile, low molecular weight molecules. □

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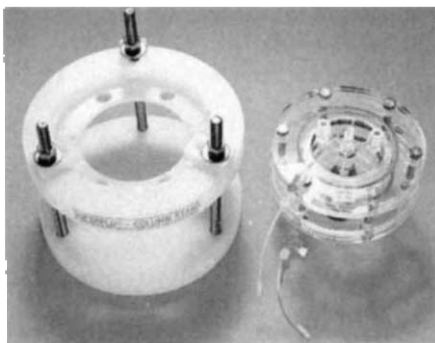
An HPLC methods development service, columns for radial-flow chromatography and software modules that turn a flatbed scanner into a scanning densitometer — new products and services within the realm of separation technology.

If you are struggling with a sticky HPLC separation problem, you may be interested to hear that LC Resources is offering a new **laboratory service for HPLC methods development** (*Reader Service No. 101*). The company says it can handle all types of samples that are amenable to HPLC separation and has broad experience in gradient methods, chiral separations, preparative separations, protein/peptide separations, and separations involving pharmaceutical and environmental samples. Some of the laboratory services being offered include: developing new methods, checking out the appropriateness of methods cited in the literature, improving methods designed for older equipment, scaling-up of methods for production, or checking out samples on new columns. All contract work is accompanied by documentation, which outlines the separation parameters and flags any potential separation problems, such as sensitivity to variations in temperature or pH. Confidentiality is ensured and all methods and documentation can be customized to suit individual user requirements.

Poretics has introduced a new line of **FilterWell-96 microtitre plates with clear, polycarbonate membrane substrates** for cell biology and other life science applications (*Reader Service No. 102*). Membrane filters offer several advantages as cell growth substrates when compared to more traditional non-porous plastic or glass substrates, says Poretics. These advantages include: free diffusion of ions and macromolecules between cells and nutrient, better cell growth rates and cell densities, anatomical and functional differentiation approaching that of the *in vivo* state, and access to both the apical and basolateral sides of the sample. When polycarbonate screen membrane filters are used as a cell growth substrate, all cells are on the surface of the membrane; this is designed to allow improved morphologi-

cal resolution with monolayer cell growth and uniform, homogeneous, confluent distribution of cells over the surface. In addition, cell interactions can be studied as different cells can be grown on each side of the polycarbonate membrane. FilterWell-96 plates are pre-sterilized and available in pore sizes of 0.2, 0.4, 3.0 and 5.0 μm with several different membrane surface characteristics.

Technicol introduces a new line of **columns for radial-flow chromatography**, which are designed to provide high-speed separations at a fraction of the cost of conventional columns (*Reader Service No. 103*). The Sepragen radial-flow columns are constructed of an outer an inner frit (the area in between containing the chromatographic media) and are compatible with soft gels or rigid media. Samples flow from the outer through to the inner frit. The larger cross-sectional area of the

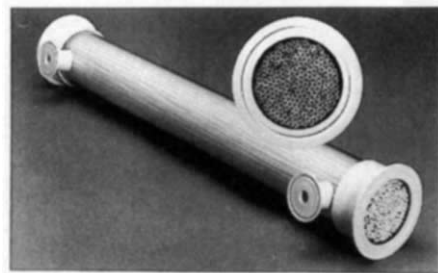


Go with the radial flow — see Technicol.

outer channel and the low bed depth allows high flow rates with little drop in pressure, Technicol says. Packing, unpacking and sterilization can be performed without taking the columns apart. The Sepragen columns, which are available in a range of sizes from 50 ml to 350 litres, are suitable for all ion-exchange, affinity, hydrophobic, reversed-phase and other forms of adsorption-desorption chromatography.

Membrane miscellany

A/G Technology has introduced a series of compact, **open-channel filtration cartridges** that are designed for processing high viscosity *Escherichia coli*, filamentous bacteria, mycelial and yeast fermentation broths, and other difficult biological/pharmaceutical separations (*Reader Service No. 104*). The TurboTube filtration cartridges are intended to optimize the energy required to achieve turbulent flow, while providing high, sta-

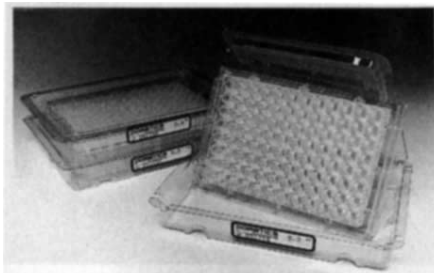


Open-channel filtration cartridges.

ble process flux rates. The open-channel membrane configuration — hollow fibre membranes in a nominal 3-mm internal diameter tubule configuration — allows independent control of the feed velocity and pressure gradient. The non-plugging design is intended to allow processing to high concentration of solids and facilitates cleaning. TurboTube membranes are available in a 0.1 μm pore size as well as a 30,000 nominal molecular weight cut-off. These membranes are provided in a range of cartridge sizes to meet laboratory, pilot- and process-scale requirements. Additional ultrafiltration molecular weight cut-offs and steam-in-place module versions are under development.

Desalt and purify microlitre samples of proteins and nucleic acids in as little as five minutes with the new **Microcon disposable microconcentrators** from Amicon (*Reader Service No. 105*). Using a standard centrifuge, samples of up to 500 μl can be reduced to as little as 5 μl . To ensure high recoveries, the Microcon devices use inverted-spin sample transfer and low-binding YM membranes. A dead-stop feature is designed to eliminate the risk of sample loss. Typical spin times are between 5 and 30 minutes. Microcon microconcentrators are available in 3,000 and 10,000 molecular weight cut-offs, although an expanded range of cut-offs will be available next month.

SpinTrex, a new benchtop, **rotary-membrane filtration system** from New Brunswick Scientific is designed to cut the time needed for protein purification from days to hours (*Reader Service No. 106*). The device pre-concentrates fermentation and cell-culture supernatants by a factor of 10 or more, substantially reducing the time needed for chromatographic purification of monoclonal antibodies and other proteins, NBS says. Rotary membrane hydrodynamics inhibits membrane fouling. And, process rates of up to 80 litres per day can be achieved.



FilterWell-96 plates with polycarbonate screen membrane cell growth substrates.

The minimal 10-ml hold-up volume also makes the processing of small batches feasible. Microprocessor control allows unattended overnight processing of large batches of 20 litres or more. A range of ultra- and micro-filters are available for applications that include biomass separations and debris removal.

Media medley

POROS P-Series media from PerSeptive Biosystems are **chromatographic supports** that are designed to provide the high resolution normally achieved with the use of HPLC/FPLC in a low-pressure format suitable for glass columns and peristaltic pumps (*Reader Service No. 107*). The company says that low-pressure HPLC offers several benefits, including high resolving power, high flow rates, minimal band spreading or loss in capacity, run times typically of less than 10 minutes, protein binding capacities of up to 90 mg ml⁻¹, and a reduced need for coldroom operation. Twenty surface chemistries are available.

Eurochrom (Knauer) announces the Eurosil Bioselect 100 chemically-bonded **stationary phases** for high performance liquid chromatography of natural and basic compounds (*Reader Service No. 108*). Eurosil Bioselect 100 is recommended as the reversed phase for separating difficult solutes: sensitive natural substances, hydrophilic and hydrophobic peptides, basic and amphoteric compounds, strongly polar components that otherwise tend to irreversible adsorption. The C8 and C18 phases, which are available in four particle sizes between 10 µ and 60 µ (irregular), can be used in HPLC, MPLC and flash LC. High sample recovery is ensured, even with sensitive substances such as basic peptides, due to 'endcapping' and special silica treatment.

Chromatography hardware

The new **online degasser** from Hewlett-Packard is designed to remove dissolved gases from HPLC solvents with an efficiency that is comparable to other degassing techniques such as the use of ultrasonics and helium gas (*Reader Service No. 109*). Although developed as a module in the HP 1050 Series modular HPLC system, the online degasser can be used as a stand-alone system with any HPLC pump or HPLC system. Dissolved oxygen forms a UV-light-absorbing complex with many solvents used in HPLC, adversely affecting the performance of UV/visible and fluorescence detectors. Dissolved gases can also affect the performance of HPLC pumps, resulting in unstable baselines or unreproducible retention times. The online degasser keeps dissolved gases at a constant low level, preventing baseline drift and ensuring optimum performance of the detector and

pump, the company says. With the online degasser, which uses a continuous vacuum process, up to four different solvents can be degassed simultaneously to constant, very low levels of oxygen at flow rates up to 10 ml min⁻¹ per channel.

The wide injection range of the new 717 **autosampler** from Waters makes the instrument suitable for analytical, microbore and preparative applications (*Reader Service No. 110*). An all-electric component design provides maximum linearity, accuracy and precision, Waters claims. Automatic needle wash routines are intended to eliminate sample loss and crossover contamination. Automated features include multimethod operations, autostandard routines for methods calibration and random-access programmability. Up to 96 samples can be preprogrammed with up to 99 injections per sample. An optional heating and cooling module provides added flexibility and can be fitted onto the back of the 717 without taking up additional bench space.

The new PC-based, high-performance **diode array LC detector** — the model L-4500 — from Hitachi includes software for not only normal data reduction but also system suitability, peak purity testing (with graphical representation), spectrum library functions, full LC system control and customized reporting (*Reader Ser-*



LC detector with 512 diodes in the array.

vice No. 111). This, together with an advanced optical design, provides a sensitivity of detection in the low parts-per-billion range (for example, less than 7 p.p.b. for anthracene on column). The L-4500 has a wavelength range of 190–800 nm with selectable spectral bandwidths of 2, 4, 7, 15 or 30 nm. Other features include spectrum displays in contour (including real-time contour display), three-dimensional, or customary chromatogram formats. When connected to a Hitachi LC system via PAN (private area network for laboratory instrumentation), the L-4500 diode array manager software can set up and control the entire LC system.

Quantitate radioactivity in HPLC eluates with the β-ram flow-through monitor from LabLogic (*Reader Service No. 112*). With β-ram, beta emitters such

as ³H to ³²P and soft gamma emitters such as ¹²⁵I can be measured at low backgrounds, typically less than 8 c.p.m., throughout the full energy spectrum. Low backgrounds are achieved by design details such as lead shielding, online chemiluminescence correction, and photomultipliers with low glass content/low voltages. Two β-ram models are available: model A, primarily intended for use with solid scintillators or for Cerenkov counting, and model B, which is equipped with a pump/mixer module for handling liquid scintillators. With the second version, the computer-controlled pump allows mixing operations to be carried out precisely, with calibrated compensation for changes in viscosity of the scintillator or the eluate. All operating parameters for the detector, splitting, mixing and data handling are set from the computer keyboard using method files that can be stored to hard disk and later down-loaded. β-ram provides three fully independent analog input channels, each capable of digitizing external detector voltage inputs. The system is designed for use with LabLogics's RaChel software. LabLogic has an alternative version of β-ram with an integral processor and memory.

Image analysis

Biometra has developed a range of software modules running under Windows that turn a computer-backed flatbed scanner into a **scanning densitometer** (*Reader Service No. 113*). The ScanPacK program is designed for routine one-dimensional (1-D) analysis of molecular weights, lengths of nucleic acid fragments, isoelectric points, banding patterns, and the quantification of peak areas (that is, the mass of individual bands). The Epson GT-6000 scanner provided with the package features three scanning wavelengths (blue, red, green) and a white light source. With three levels of sensitivity and seven different light intensities, ScanPacK is optimized for the analysis of 1-D electrophoresis — especially immunoblots that are rather insensitive to red and white light. Besides the densitograms, the scanned images can be exported (with publication-quality) to laser printers, thus avoiding the need for processing in a darkroom. With the backing of an overhead light source, Biometra says the scan-



ScanPacK densitometry software.

ner can reproduce the most dense autoradiographs and silver-stained gels; this allows for quantification even when the densities of spots and the background are very close. ScanPacK II includes an additional two-dimensional extension that allows for background reduction, quantification of spots, and characterization of spots according to their isoelectric points and molecular weights. All data can be

exported to spreadsheets, graphics or desktop publishing programs.

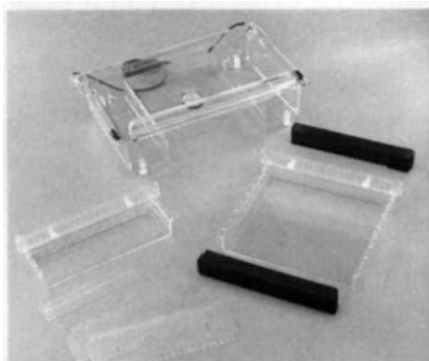
BASys is a high-resolution **image analysis system** from Biotec-Fischer for the quantitative and qualitative analysis of electrophoretic separations and similar objects (*Reader Service No. 114*). The system consists of three groups of hardware: image pickup, data processing and data output. At the heart of the image pickup is a high-sensitivity CCD camera. Two models are available: the standard KP-161 model and the VL-350 model for very faint objects. The CCD cameras generate a grey image in real time. This grey image encompasses 256 grey levels and serves as the basis for all subsequent operations. A camera stand and daylight transilluminator complete the image pickup system. An IBM-compatible 386 computer is used as the data processing unit. A specially designed frame-grabber module allows interactive image processing of the captured grey image, without using the working storage area of the computer. Several software programs are available for use with BASys. These include programs for one- and two-dimensional analysis, DNA fingerprint analysis and dot-blot analysis. Additional program modules can be purchased for curve subtraction, molecular weight determination, external standards, geometrical image distortion and DNA fingerprint database.

Isco's Model 3850 personal capillary electrophoresis (CE) system is now available with a new **controller for electromigration (EMI)** and vacuum injection of nanolitre samples (*Reader Service No. 115*). The EMI mode allows separation of DNA fragments using gel-filled capillaries. EMI and vacuum modes also improve quantitative accuracy in a full range of capillary electrophoresis methods, including MECC, gel and conven-

tional free-solution methods with most coated or non-coated capillary columns. The new injector complements the 3850's split-flow injection, which provides an easy interface for CE-mass spectrometry and other post-column detection. Other features of the CE system include a 30-kV, reversible-polarity power supply and 190 to 360 nm variable UV detection with femtomole sensitivity.

Gel boxes

For the rapid screening of a large number of DNA or RNA fragments in agarose gels, E-C Apparatus recommends its EC360 Maxicell **submarine gel system** (*Reader Service No. 116*). Each device is



E-C Apparatus' Maxicell is on the level.

supplied with one 20 × 20-cm and one 10 × 20-cm gel tray, two comb holders, and two each of the following: 40-well, 22-well, 10-well and 2-well combs. Other design features include a one-piece, injection-moulded construction for leak-proof operation, recirculation ports, and a built-in level and levelling feet. The 20 × 20-cm gel tray is equipped with a UV-fluorescent centimetre rule.

Jordan Scientific's QuickCast Gel Casting Clamps provide **tapeless gel casting** and also ensure that gels are cast evenly because each clamp has a built-in levelling system (*Reader Service No. 117*). Evenly-cast gels provide better reproducibility, gel after gel, Jordan says. The QuickCast Gel Casting System is now included as standard with all of the company's submarine gel systems, such as the OnePhorAll self-recirculating gel systems.

The new **sequencing gel system** from Whatman can be used to run large numbers of samples (up to 78) with high resolution (*Reader Service No. 118*). The sequencing system has an extended upper buffer chamber that provides even heat distribution across the entire gel. Both the upper and lower buffer chambers have an in-line clean-up. □

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